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High prevalence and breast cancer predisposing role of the BLM c.1642 C>T (Q548X) mutation in Russia

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The *BLM* gene belongs to the RecQ helicase family and has been implicated in the maintenance of genomic stability. Its homozygous germline inactivation causes Bloom syndrome, a severe genetic disorder characterized by growth retardation, impaired fertility and highly elevated cancer risk. We hypothesized that *BLM* is a candidate gene for breast cancer (BC) predisposition. Sequencing of its entire coding region in 95 genetically enriched Russian BC patients identified two heterozygous carriers of the *c.1642 C>T* (Q548X) mutation. The extended study revealed this allele in 17/1,498 (1.1%) BC cases *vs.* 2/1,093 (0.2%) healthy women ($p = 0.004$). There was a suggestion that *BLM* mutations were more common in patients reporting first-degree family history of BC (6/251 (2.4%) *vs.* 11/1,247 (0.9%), $p = 0.05$), early-onset cases (12/762 (1.6%) *vs.* 5/736 (0.7%), $p = 0.14$) and women with bilateral appearance of the disease (2/122 (1.6%) *vs.* 15/1376 (1.1%), $p = 0.64$). None of the *BLM*-associated BC exhibited somatic loss of heterozygosity at the *BLM* gene locus. This study demonstrates that *BLM* Q548X allele is recurrent in Slavic subjects and may be associated with BC risk.

Cancer Genetics

Hereditary risk factors are strongly implicated in breast cancer (BC) predisposition. Mutations in *BRCA1* and *BRCA2* genes account for approximately 15–20% of familial BC clustering among first degree relatives.[1,2] It was assumed for some years that a significant share of BC predisposition can be explained by common low-penetrance single nucleotide polymorphisms (SNPs). Recent technological advances permitted to run several genome-wide association studies involving large collections of BC patients and controls. Several highly reproducible SNP–BC associations have been identified. However, the per-allele odds ratios (ORs) for BC-associated SNPs vary between just over 1 to around 1.30.[3] Although the power of genome-wide association studies is limited and therefore some more BC-associated SNPs will possibly be identified in the future, it is getting apparent that most of the excess familial risk of BC still remains unexplained.

Key words: BLM, Bloom syndrome, breast cancer, BRCA1, loss of heterozygosity

Abbreviations: BC, breast cancer; CI, confidence intervals; ER, estrogen receptor; HRMA, high-resolution melting analysis; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable; OR, odds ratio; PR, progesterone receptor; SNP: single nucleotide polymorphism

Additional Supporting Information may be found in the online version of this article

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Table 1. Characteristics of the study subjects

Subjects	Number	Mean age (age range), years	Patients with clinical indicators for BC predisposition[1], *n* (%)
Breast cancer patients	1,498	51.0 (24–81)	924 (61.7%)
Consecutive	879	54.0 (25–81)	437 (49.7%)
Genetic counseling	451	45.3 (24–78)	386 (86.6%)
Collected at random	168	51.2 (26–80)	101 (60.1%)
Healthy females	1,093	42.0 (18–74)	na
Healthy males	1,091	36.4 (18–74)	na

[1]1st degree family history of BC, bilaterality of the disease, or age at onset $\leq$ 50 years; na: not applicable

The critical limitation of genome-wide association studies is the inability to pinpoint rare disease-associated alleles which confer moderate to high risks of the disease. These rare mutations can be detected through other approaches, *e.g.*, linkage analysis, sequencing of candidate genes and whole genome sequencing. Linkage studies have failed to reveal BC-predisposing genes other than *BRCA1* and *BRCA2*, suggesting that there is no single major undiscovered contributor left. Sequencing of potentially important genes in *BRCA1/2*-negative familial BC cases has already led to identification of a number of novel contributors, including *CHEK2, PALB2, BRIP1, RAD50, etc.*[2] It is important to acknowledge that distinct ethnic groups have distinct spectrum of BC-predisposing mutations, depending on the genetic background of their founders.[4–6] Therefore, a systematic DNA analysis of hereditary BC in yet unstudied populations is particularly likely to reveal new determinants of cancer susceptibility.

In order to search for novel BC genes, we have collected a set of 95 BC patients who show clinical evidence for hereditary nature of their disease but are nevertheless negative for known Russian BC-predisposing mutations. We sequenced genes involved in various aspects of genome maintenance and detected two *BLM c.1642 C>T* (Q548X) mutation carriers within this genetically enriched subset of BC cases. A subsequent case–control study revealed that the *BLM* Q548X mutation is recurrent in the Russian population and suggested its role in BC predisposition.

Material and Methods

One thousand four hundred ninety-eight unrelated BC patients referred to the N.N. Petrov Institute of Oncology (St.-Petersburg, Russia) were available for the analysis. Eight hundred seventy-nine out of 1,498 BC were consecutive cases collected within April 2001 to February 2002, March 2003 to January 2004, June 2006 to May 2007 and March 2008 to May 2008. Another 451 BC were referred to the laboratory within years 2008–2010 specifically for genetic testing, because of either clinical signs of hereditary BC or personal concerns of the patient. The remaining 168 BC cases were collected at random. Main characteristics of the study subjects are provided in Table 1.

Ninety-five DNA samples from the total BC collection were selected for the candidate gene sequencing. These cases (mean age: 42.2 years; age range: 24–77 years) showed signs of genetic predisposition to BC, but were negative for mutations in the coding region of *BRCA1* gene as well as for the founder mutations in *CHEK2, NBS1* and *BRCA2* genes.[7,8] Forty-three (45%) of these women had single clinical feature of hereditary BC (early onset: 29; bilaterality: 14); 48 (51%) cases possessed combination of two clinical indicators of BC predisposition (early onset and affected first-degree relative(s): 32; bilaterality and affected first-degree relative(s): 2; bilaterality and early onset: 14); the remaining 4 (4%) females had combination of all three clinical indicators of BC susceptibility.

Tumor-free subjects (controls) included 1,093 females (mean age: 42.0 years; age range: 18–74 years) and 1,091 males (mean age: 36.4; age range: 18–74 years); they were recruited from the blood transfusion units of the N.N. Petrov Institute of Oncology ($n = 744$) and the City Hospital No. 26 ($n = 1074$) as well as at various departments of the City Hospital No. 2 ($n = 281$) and Institute of Pulmonology, St.-Petersburg ($n = 85$).

All study participants were of white European ancestry, had predominantly Slavic origin and were recruited between 1998 and 2011 in the city of St.-Petersburg. The study was approved by the local Ethical Committee.

The analysis of entire coding region of the *BLM* gene was performed using high-resolution melting analysis (HRMA) followed by direct sequencing of abnormally melted fragments.[6] PCR amplifications were set up in the presence of EvaGreen dye (Biotium, Hayward, CA). PCR reactions and HRMA were carried out using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer sequences are presented in the Supporting Information Table 1.

Genotyping for the *c.1642 C>T* (Q548X) mutation was performed by HRMA using the available mutation-positive and -negative samples as control. All identified mutations were confirmed by DNA sequencing (Fig. 1). Microdissected archival tumor samples from Q548X mutation carriers were used for analysis of somatic mutations. Loss of heterozygosity (LOH) in the *BLM* gene locus was assessed by SYBR Green

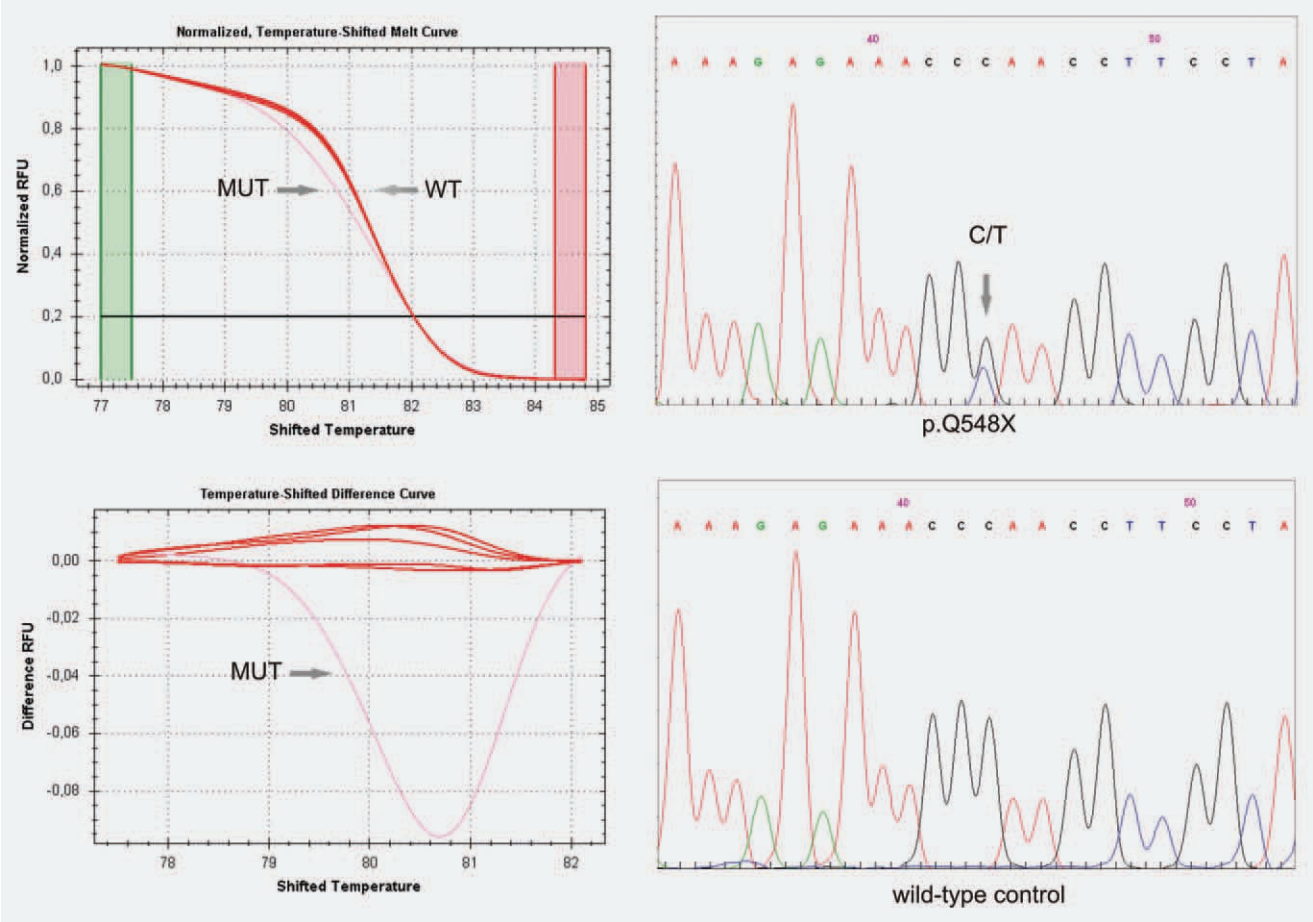

Figure 1. *BLM c.1642 C>T* (Q548X) mutation identified by high-resolution melting and sequencing analysis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

based allele-specific PCR.[9] Primers were ATGACTTAGAAA GAGAAACCC (wild-type allele), ATGACTTAGAAAGA GAAACCT (mutated allele) and TGATGGGTTGATAGG CAGC (common primer). Mutations in the *BLM* poly(A)9 tract were analyzed by direct DNA sequencing.[10] Microsatellite instability (MSI) status was evaluated using *BAT26* quasi-monomorphic marker.[11] Immunohistochemical (IHC) assessment of the tumors was carried out using Novocastra (Newcastle on Tyne, UK) antibodies for ER (clone 6F11), PR (clone 16) and EGFR (clone 25), and DAKO (Carpinteria, CA) antibodies for HER2 (HercepTest) and CK 5/6 (clone D5/16 B4). IHC classification for BC expression subtypes was performed as described in Ref. [12].

Mutation frequencies in cases and controls were compared using the Fisher exact test statistic. All tests were two-tailed. Statistical analysis was performed using the SPSS 10 software package.

Results

Sequencing of the entire coding region of the *BLM* gene revealed two heterozygous carriers of clearly inactivating mutation among 95 genetically enriched BC cases. Both *BLM* heterozygotes contained the *c.1642 C>T* (Q548X) allele. The Q548X mutation has been described earlier by the Bloom syndrome registry, but its ethnic origin remained unknown.[13] Interestingly, both Q548X carriers but none of the remaining 93 women had another sequence variation in the *BLM* gene, *c.2119 C>T*. If present alone, this event would result in the P707S substitution; however, given that the *c.2119 C>T* is located downstream to the stop-codon generating mutation *c.1642 C>T*, it cannot be translated into the protein sequence change. One BC patient was heterozygous for previously unknown aminoacid substitution, c.3416 G>C (R1139P). The potential effect of this variation on the protein properties was assessed using available software tools (PANTHER: http://www.pantherdb.org/tools/csnpScore.do; Pmut: http://mmb.pcb.ub.es/PMut/; PolyPhen: http://genetics.bwh.harvard.edu/pph/), and all these predictive algorithms suggested functional significance of this SNP. Other SNPs identified upon *BLM* gene sequencing have already been described in the NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and included *c.1722A>G* (L574L, rs28385011, 2/95 (2%)), *c.3531C>A* (A1177A, rs2227934, 1/95 (1%)), *c.3102G>A* (T1034T, rs2227933, 24/95 (25%)) and *c.2603 C>T* (P868L, rs11852361, 8/95 (9%)).

The analysis of the Q548X allele was extended to all available BC patients, and revealed this mutation in 17/1,498

Table 2. Frequency of *BLM* Q548X mutation carriers in cancer patients and healthy controls

Study groups	*BLM* Q548X carriers
Breast cancer patients (all cases)	17/1498 (1.1%)
Bilateral BC patients	2/122 (1.6%)
Unilateral BC patients	15/1376 (1.1%)
Age of diagnosis[1]	
≤50 years	12/762 (1.6%)
≥51 years	5/736 (0.7%)
Family history	
Positive	6/251 (2.4%)
Negative	11/1247 (0.9%)
Clinical indicators of hereditary BC	
Family history or bilaterality or early onset	14/924 (1.5%)
None	3/574 (0.5%)
Mode of patients selection	
Consecutive	10/879 (1.1%)
Genetic counseling	5/451 (1.1%)
Random	2/168 (1.2%)
Healthy controls	
Females	2/1093 (0.2%)
Males	3/1091 (0.3%)

[1]For the bilateral breast cancer patients, age of diagnosis of the first tumor.

(1.1%) cases; in comparison, only 2/1,093 (0.2%) healthy females carried this genetic defect ($p = 0.004$) (Table 2). Odds ratio calculated on the basis of comparison of consecutive BC cases (10/879 (1.1%)) *vs.* unaffected women approached 6.28 (95% confidence intervals (CI): 1.52–42.18). Genotyping of healthy males identified 3/1,091 (0.3%) subjects with the Q548X mutation. All *c.1642 C>T* (Q548X) heterozygotes also carried the *c.2119 C>T* substitution indicating close linkage of these mutations.

Distribution of the Q548X heterozygotes within distinct subsets of BC patients favored its involvement in BC predisposition. Mutation carriers tended to be more common in patients reporting first-degree family history of BC (6/251 (2.4%) *vs.* 11/1247 (0.9%), $p = 0.05$), early-onset cases (12/762 (1.6%) *vs.* 5/736 (0.7%), $p = 0.14$) and women with bilateral appearance of the disease (2/122 (1.6%) *vs.* 15/1376 (1.1%), $p = 0.64$) (Table 2). Genetic material from the BC-affected relative was available for 1 *BLM* mutation carrier (BC1158, Supporting Information Table 2); genotyping revealed that the mother of this patient BC1158 was heterozygous for the Q548X allele as well. 1,129/1,498 BC patients included in the study were evaluated for the presence of inactivating germline *BRCA1* mutations either by the founder mutation test ($n = 949$) or by the analysis of the entire coding region of the gene ($n = 180$),[7] and 69 were revealed to have an altered copy of the *BRCA1*; 1 of these patients turned out to be a combined heterozygote, containing both *BRCA1 5382insC* and *BLM c.1642 C>T* alleles.

Clinical characteristics of *BLM*-related BC are presented in Supporting Information Table 2. Patients with the Q548X mutation had usual TNM and grade distribution. IHC data were available for 18 carcinomas (Supporting Information Table 2). Expression of steroid hormone receptors was detected in 13/18 (72%) cases; the majority of these tumors did not express basal markers or high amounts of HER2, and therefore appeared to belong to the Luminal A expression subtype. 5/18 (28%) BC showed the Basal-like expression patterns; one of these cancers belonged to the combined *BRCA1/BLM* mutation carrier. Five women were treated by neoadjuvant therapy and therefore could be accessed for tumor chemosensitivity; three patients showed nearly complete pathological response and two experienced partial clinical response.

Somatic loss of the remaining allele is regarded as a canonical mechanism for tumor-specific inactivation of hereditary cancer genes.[14] Eleven microdissected tumor samples were successfully analyzed for LOH at the *BLM* gene locus, and none of those had deletion of the *BLM* allele (data not shown). Interestingly, tumor from the combined *BRCA1/BLM* mutation carrier retained heterozygosity at both *BRCA1* and *BLM* loci (Supporting Information Table 2). The coding region of the *BLM* gene contains poly(A)9 track, which is frequently targeted by somatic mutations in microsatellite unstable cancers.[10] We performed sequencing analysis of 14 tumor-derived DNA samples from *BLM* mutation carriers but this revealed no alterations at the poly(A)9 repeat (data not shown). Some tumors arising in patients with Bloom syndrome were shown to have microsatellite instability.[15] We used *BAT26* quasimonomorphic marker for the microsatellite status assessment in 15 *BLM*-related BC, and detected no instances of MSI (data not shown).

Discussion

The *BLM* gene is located on chromosome 15q26.1. It encodes 1,417 aminoacid product belonging to the RecQ helicase family. The BLM protein plays multiple roles in DNA maintenance.[16] It senses DNA damage and recruits other repair proteins to the site of DNA lesion.[17] The BLM helicase ensures fidelity of homologous recombination by dissolving double Holliday junctions and thus preventing crossing-over between sister chromatids.[18] In addition, it directs regression of stalled replication fork.[19] Finally, BLM may participate in telomere maintenance.[20] Germline *BLM* inactivation causes the rare human disorder, Bloom syndrome. This disease is characterized by severe growth retardation, characteristic skin erythema, sun hypersensitivity, male infertility and female subfertility, immunodeficiency, and, most remarkably, pronounced predisposition to a wide spectrum of various malignancies. Individuals with Bloom syndrome usually succumb to neoplastic disease in the third decade of life, and cancer is regarded as the main cause of their premature mortality.[16,21]

Table 3. *BLM* heterozygosity in breast cancer patients and healthy controls

Study	BC patients	Controls	OR (95% CI)	*p* Value
Gruber et al.[25]	5/375 (1.3%)	14/1839 (0.8%)	1.76 (0.57–4.78)	0.350
Cleary et al.[26]	4/294 (1.4%)	8/944 (0.8%)	1.61 (0.42–5.37)	0.494
This study*	10/879 (1.1%)	2/1093 (0.2%)	6.28 (1.52–42.18)	0.007
Mantel-Haenszel meta-analysis			2.49 (1.29–4.78)	0.006

*Only consecutive BC cases were considered for OR calculation.

Cells from Bloom syndrome individuals demonstrate highly elevated level of chromosomal instability, which is manifested by multiple sister chromatid exchanges, chromosomal breaks, translocations, *etc.*[22,23]

BLM heterozygous individuals have normal phenotype, although some laboratory tests occasionally reveal evidence for subtle genomic instability in these subjects.[24] Gruber *et al.*[25] reported increased risk of colorectal cancer in *BLM* mutation carriers, however this finding was later disputed by Cleary *et al.*[26] Cancer-predisposing role of *BLM* heterozygosity is also supported by studies in genetically engineered mice.[27]

This investigation has demonstrated an excess of germline *BLM* mutations in BC patients as compared to healthy women (Table 2). Furthermore, *BLM* heterozygosity tended to be more prevalent in BC cases characterized by clinical signs of hereditary disease, such as first-degree family history of BC, early onset and bilaterality of the disease (Table 2), although the numbers were too small to assess this reliably. The use of hospital-based collection of BC cases represents a limitation of the study. The N.N. Petrov Institute of Oncology is regarded in the city of St.-Petersburg as a clinic specializing in the management of hereditary forms of cancer, there may therefore be a referral bias towards patients with familial and/or early-onset and/or bilateral BC even for consecutive BC cases. Consequently, the OR = 6.28 (95% CI: 1.52–42.18) calculated in this study is likely to be an overestimate of true relative risk conferred by the Q548X allele. Two prior case–control studies compared the frequency of *BLM* mutations in BC patients and unaffected controls, and both observed a trend towards association between *BLM* heterozygosity and the disease risk (Table 3); these reports did not evaluate relationships between *BLM* germline status and clinical characteristics of BC.[25,26]

BLM-related breast carcinomas did not display LOH at the *BLM* locus. Although earlier studies described the loss of the remaining wild-type allele as consistent feature of *BRCA1/2*-related tumors,[28,29] the role of somatic LOH in *BRCA*-driven breast carcinogenesis has been disputed by recent investigations.[14,30] Loss of the normal allele is also not a characteristic for breast tumors arising in *CHEK2, NBS1* and *RAD50* germline mutation carriers,[31–33] and there is conflicting evidence regarding the LOH status in *PALB2*-associated BC.[34,35] It has been hypothesized that other mechanisms of somatic inactivation of these genes may occur, or, alternatively, a systemic haploinsufficiency caused by decreased gene dosage facilitates accumulation of tumor-specific oncogenic mutations.[36,37]

The present study detected one BC patient with combined heterozygosity for *BRCA1* and *BLM* mutations. Notably, the tumor tissue from this patient retained heterozygosity both at *BRCA1* and *BLM* loci, although its basal-like features favored a dominant role for *BRCA1* in the cancer appearance (Supporting Information Table 2). As LOH test was performed on microdissected tumor sample, failure to detect allelic loss cannot be explained by the presence of the normal cells in the analyzed material. Combined heterozygosity involving *BRCA1* germline inactivation has been repeatedly described for *BRCA2, CHEK2, NBS1* and *BLM* mutation carriers.[9,38–40] Surprisingly, subjects with combined cancer-predisposing alleles do not have evident multiplication of cancer risk and often remain healthy in their mid-life.[9,39,40]

The Bloom syndrome registry describes several dozens of various mutations in the Bloom gene, and many of them appear to be recurrent.[13] However, elevated ethnic-specific incidence of *BLM* heterozygosity has been reported only for Ashkenazi Jews, for whom the frequency of the *c.2207_2212delATCTGAinsTAGATTC* (BLM^{Ash}) allele approaches to 0.4–1%.[41] High frequency of the *BLM c.1642 C>T* (Q548X) mutation observed in the present study implies its possible founder role in Slavic populations. People of Slavic descent populate Russia, Poland, Ukraine, Belarus, Lithuania and some other European countries, as well as constitute a substantial fraction of the inhabitants in North America. Slavic populations are characterized by a surprisingly strong founder effect.[7,8,42,43] Further studies on the distribution of the Q548X allele in various geographic regions may have important implications for the Bloom syndrome diagnosis and prevention.

This report provides evidence for the role of *BLM* heterozygosity in BC risk. It justifies an analysis for *BLM* founder mutations in Ashkenazi and Slavic case–control series as well as sequencing of this gene in familial BC samples from other ethnic groups.

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