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EPIDEMIOLOGY

Exome sequencing study of Russian breast cancer patients suggests a predisposing role for *USP39*

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Abstract

Purpose Germline variants in known breast cancer (BC) predisposing genes explain less than half of hereditary BC cases. This study aimed to identify missing genetic determinants of BC.

Methods Whole exome sequencing (WES) of lymphocyte DNA was performed for 49 Russian patients with clinical signs of genetic BC predisposition, who lacked Slavic founder mutations in *BRCA1, BRCA2, CHEK2*, and *NBS1* genes.

Results Bioinformatic analysis of WES data was allowed to compile a list of 229 candidate mutations. 79 of these mutations were subjected to a three-stage case–control analysis. The initial two stages, which involved up to 797 high-risk BC patients, 1504 consecutive BC cases, and 1081 healthy women, indicated a potentially BC-predisposing role for 6 candidates, i.e., *USP39* c.*208G > C, *PZP* p.Arg680Ter, *LEPREL1* p.Pro636Ser, *SLIT3* p.Arg154Cys, *CREB3* p.Lys157Glu, and *ING1* p.Pro319Leu. *USP39* c.*208G > C was strongly associated with triple-negative breast tumors ($p = 0.0001$). In the third replication stage, we genotyped the truncating variant of *PZP* (rs145240281) and the potential splice variant of *USP39* (rs112653307) in three independent cohorts of Russian, Byelorussian, and German ancestry, comprising a total of 3216 cases and 2525 controls. The data obtained for *USP39* rs112653307 supported the association identified in the initial stages (the combined OR 1.72, $p = 0.035$).

Conclusions This study suggests the role of a rare splicing variant in *BC* susceptibility. *USP39* encodes an ubiquitin-specific peptidase that regulates cancer-relevant tumor suppressors including CHEK2. Further epidemiological and functional studies involving these gene variants are warranted.

Keywords Hereditary breast cancer · Non-BRCA1/2 · Germline mutations · Whole exome sequencing · Case–control study

Abbreviations

BC	Breast cancer
WES	Whole exome sequencing
HRM	High resolution melting (HRM)
AS-PCR	Allele-specific PCR
LOH	Loss-of-heterozygosity

Introduction

Breast cancer (BC) is the most common oncological disease among women [1]. A significant portion of BC incidence is attributed to hereditary predisposition to the disease. A number of highly or moderately penetrant BC-associated genes have been discovered in the past, including *BRCA1, BRCA2, TP53*, *PTEN*, *STK11*, *PALB2, CHEK2, ATM,* and additional candidate susceptibility genes such as *BARD1, NBN, BLM, RAD51C, RAD51D, XRCC2, FANCM, MRE11A* etc. [2–12]. Nevertheless, even comprehensive analysis of all known BC genes would be capable to find definite genetic cause of the disease only in 20–30% of women with overt clinical features of hereditary cancer syndrome [13–15].

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There are ongoing investigations aimed to identify novel BC-predisposing genes.

The first BC gene-seeking studies focused on extensive BC pedigrees. This approach turned out to be extremely successful, as exemplified by the discovery of *BRCA1* and *BRCA2* genes. However, collection of multiple-case families is highly complicated in communities with low birth rate, especially in countries that experienced historical turbulences in the past. Furthermore, family-based studies are capable to identify mainly the genes with very high penetrance; however, they may have limited capacity in revealing moderately penetrant but still medically relevant gene candidates. Therefore, it is common to use clinical surrogates of BC predisposition, such as early-onset and bilateral BC disease, to enrich for hereditary breast cancer [16].

Hereditary cancer studies are significantly compromised by genetic heterogeneity of population. Founder communities provide significant advantage in this respect; indeed, if a given gene plays a role in predisposition to a certain disease, its pathogenic alleles are usually represented by a few recurrent variants. Validation of newly identified gene candidates can then be more easily achieved via rapid and cost-efficient case–control studies. Importantly, some Slavic countries (Poland, Russia, Ukraine, and Belarus) demonstrate highly pronounced founder effects. This is exemplified by a high frequency of certain recurrent BC-predisposing mutations e.g., in *BRCA1*, *CHEK2*, or *ATM* genes as well as clinically relevant pathogenic alleles for some other diseases (e.g., *SCO2* c.418G > A; *GJB2* c.35delG) [17–20].

We assumed that the application of exome analysis to genetically enriched Slavic BC patients will allow to identify novel BC-predisposing genes. In the present study, we therefore performed exome sequencing and subsequent case–control studies on Russian patients with clinical evidence of hereditary breast cancer.

Materials and methods

We initially included in the study 49 Russian women with BC, who demonstrated clinical signs of hereditary disease, but were lacking founder mutations in *BRCA1, BRCA2, CHEK2*, and *NBN* genes (Supplementary Table S1). We also analyzed 18 cancer-free controls to facilitate the exclusion of nonrelevant candidates. Exome capture was performed using Nextera Rapid Capture Exome kit (Illumina, USA). Whole exome sequencing (WES) was performed using either Illumina MiSeq (16 samples) or Illumina NextSeq platform (33 samples) with the mean depth of coverage 36× for MiSeq and 81× for NextSeq (Fig. 1).

The obtained paired-end reads were aligned to the reference genome (GRCh37/hg19) using the MEM algorithm of BWA software v.0.7.15-r1140 [https://doi.org/10.1093/bioinformatics/btp324] and were stored in BAM files by Samtools v.1.7 [https://doi.org/10.1093/bioinformatics/btp352]. Duplicate reads were marked by Picard tools v.2.9.0 [http://broadinstitute.github.io/picard]. Variants for each sample were called separately using HaplotypeCaller walker of Genome Analysis Toolkit (GATK) v.3.6 according to the GATK Best Practices work flow [https://doi.org/10.1002/0471250953.bi1110s43]. We required a minimum depth of 10 and quality greater than 50 as prefilters. Single-sample variant files were normalized, merged, and saved to the multi-sample VCF-file by bcftools v.1.7 [https://doi.org/10.1093/bioinformatics/btr509]. The multi-sample file was annotated using a SnpEff v.4.3t tool [https://doi.org/10.4161/fly.19695] and variants with predicted high or moderate impact were selected for further consideration.

The criteria for variant filtering are presented in Fig. 2. The selected candidates were subjected to manual inspection in the Integrative Genomics Viewer (IGV) browser [http://www.broadinstitute.org/igv/home]. Sanger sequencing was applied to primary DNA samples ("index" cases) in order to validate newly identified variants.

The BC-predisposing role of candidate mutations/genes was evaluated using 2-stage case–control analysis (Supplementary Fig. S1). All BC patients included in the study were negative for common Slavic founder mutations [*BRCA1:* c.5266dupC (5382insC), c.4034delA (4153delA), c.68_69delAG (185delAG); *BRCA2* c.5946delT (6174delT); *CHEK2:* c.1100delC (1100delC), c.444 + 1G > A (IVS2 + 1G > A); *NBN* c.657_661delACAAA (657del5)]. The group of high-risk BC patients (median age: 43 years; range: 23–79 years) was represented by 797 women, who were forwarded to the N.N. Petrov Institute of Oncology (St.-Petersburg, Russia) between the years 2008–2018 specifically for genetic testing and had at least one clinical indicator of BC predisposition (1st degree family history of BC, bilaterality of the disease, age at onset ≤ 50 years). 1504 consecutive BC patients (median age: 57.0 years; range: 24–90 years) were recruited in the N.N. Petrov Institute of Oncology (time intervals: April 2001–February 2002, March 2003–January 2004, June 2006–May 2007 and March 2008–May 2008), St.-Petersburg Regional Cancer Hospital (February 2015–June 2015), and St.-Petersburg City Cancer Center (February 2017–April 2017). 132/1504 (8.8%) consecutive patients reported a 1st degree family history of BC and/or ovarian cancer; 396/1504 (26.3%) were diagnosed by the age ≤ 50 years; 54/1504 (3.6%) had multiple primary cancers (Supplementary Table S1). Cancer-free controls were collected at random and had a median age of 44 years (range: 21–82 years).

Our initial evaluation of candidate alleles involved an average 385 high-risk BC patients (range: 150–656) and 330 healthy middle-aged women (range: 150–633) (Supplementary Fig. S1). Promising gene variants were

Fig. 1 Work flow for the detection of BC-predisposing mutations using whole exome sequencing (WES)

further assessed in the extended case–control analysis, which included an average 1330 consecutive BC patients (range: 1220–1504), 356 additional high-risk BC patients (range: 203–412), and 716 additional healthy female controls (range: 681–751). The sample size of the study varied from gene to gene; exact numbers of analyzed subjects are given in Supplementary Table S5.

Candidate genetic variants were genotyped by high resolution melting (HRM) analysis followed by Sanger sequencing of abnormally melted DNA fragments or by real-time allele-specific PCR (AS-PCR). The results of case–control analysis were statistically analyzed by SPSS software (version 22) using two-sided Fisher's exact or Chi-square test.

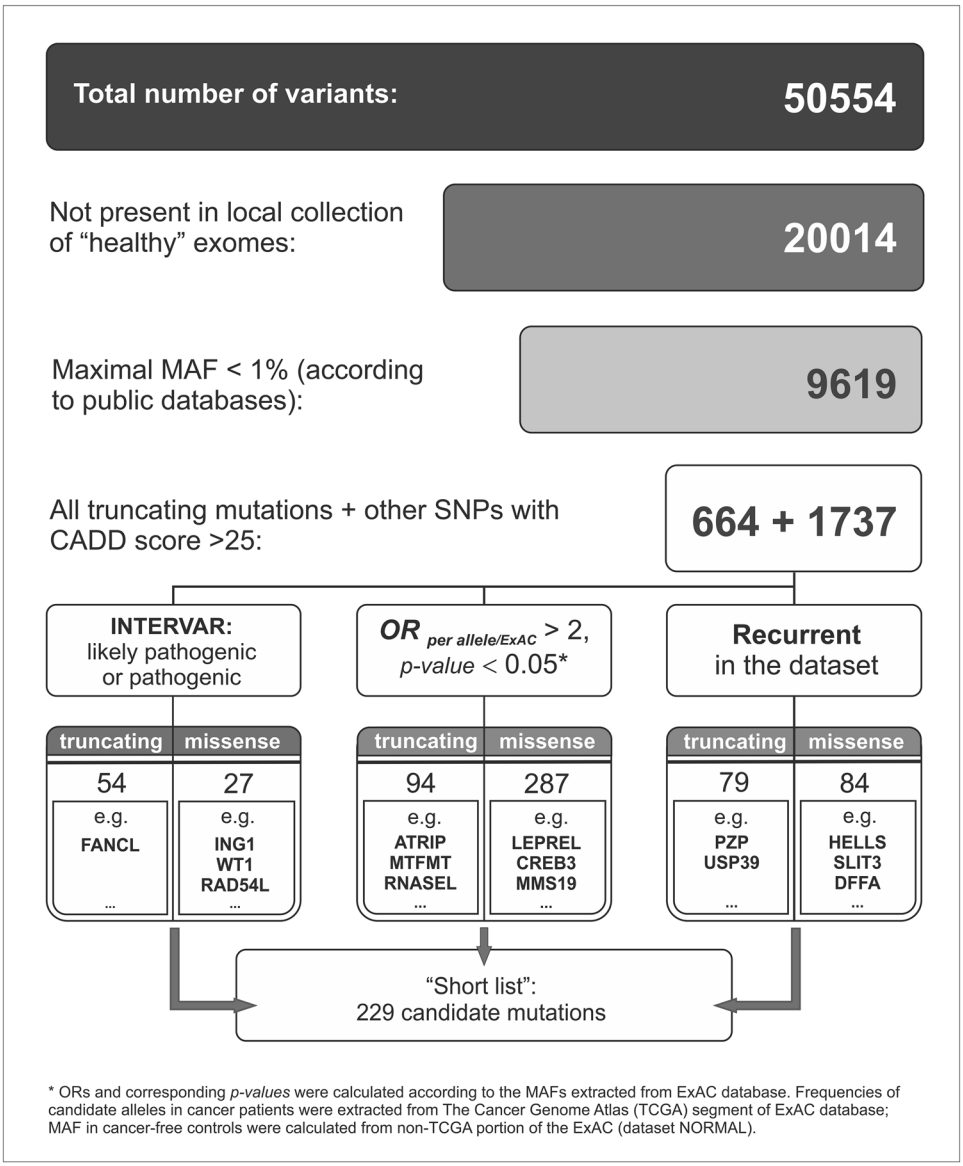

Fig. 2 WES data analysis: filtration and prioritization strategy

For replication of significant associations, we used the three breast cancer case–control series of the Hannover–Minsk Breast Cancer Study (HMBCS), the Hannover–Ufa Breast Cancer Study (HUBCS), and the Hannover Breast Cancer Study (HaBCS). All three studies have previously been described [17, 21]. In brief, the HMBCS consists of 1891 breast cancer patients recruited in the Republic of Belarus during the years 1998–2007, and 1019 healthy volunteers from the same population who had no personal history of breast cancer at the time when entering the study. The HUBCS consisted of breast cancer patients unselected for family history who were living in the Volga Ural region of Russia and diagnosed during the years 2000–2007 at the oncological center in Ufa (Bashkortostan) and 542 volunteers from the same geographic regions. The HaBCS consists of over 1000 unselected German breast cancer patients and 1013 healthy females who were living in the Lower Saxonian region of Germany and have been recruited at the Gynecology Research Unit of Hannover Medical School.

In the replication study, genotyping was carried out with allele-specific SNP-type assays using 192.24 Dynamic Arrays on a Biomark Real-time PCR platform according to the manufacturer's instructions (Fluidigm Corp.). Cluster plots were automatically called, with manual adjustments wherever necessary and case–control data were analyzed using logistic regression analysis with STATA 12. A fixed-effects meta-analysis of the three different case–control studies was run using the *metan* command in STATA.

Wherever possible, tumor tissues obtained from the carriers of presumably BC-predisposing mutations were subjected to the loss-of-heterozygosity (LOH) analysis. Somatic deletions of the remaining allele were evaluated

by allele-specific PCR and Sanger sequencing as described in [22].

Results

Forty-nine BC patients with clinical signs suggestive of hereditary disease were analyzed through whole exome sequencing (WES). Given that these patients were tested only against recurrent Slavic cancer-predisposing mutations, this analysis expectedly led to the identification of a number of known BC risk alleles. In particular, 21 DNA samples carried mutations in *BRCA1, BRCA2, PALB2, BLM, RAD51C, RAD50, RAD54L, FANCM, WRN, MMS22L*, and *ERCC4* genes with predicted pathogenicity (Supplementary Table S2). WES analysis of the remaining 28 cases identified 50,554 non-silent variants (Supplementary Table S3). We further filtered out alleles, which demonstrated population frequency above 1% (ExAC database) or were present in our collection of 18 exomes obtained from cancer-free controls. This permitted us to compose a list containing 9619 rare mutations. We included into the further analysis only protein-truncating variants ($n=664$) and missense variants with CADD score > 25 ($n=1737$). Variant filtering is described in Fig. 2.

We manually screened the list of these 2401 alleles and considered them as deserving attention if they met one of the below-described criteria. In particular, we prioritized allelic variants defined as "pathogenic/likely pathogenic" by the ACMG-guided scoring system INVERVAR. This five-tier categorization system uses a total of 28 criteria based on different sources of data such as population frequencies, in silico analysis, functional experiments, and segregation data [23, 24].

We also compared the frequency of mutations in the ExAC cancer cohort versus cancer-free population and selected for the study the alleles producing $OR_{\text{per allele}} > 2$ at $p < 0.05$ [25, 26]. Irrespective of the potential gene function, we also selected variants which occurred in our BC exome collection twice but appeared exceptionally rare or absent in the general population; we reasoned that this frequency (2/28, 7%) is compatible with the frequency of known highly penetrant pathogenic alleles in high-risk BC cases (e.g., *BRCA1* c.5266dupC (5382insC)) [27]; *CHEK2* c.1100delC [20], etc.). Cancer-relevant functions of the candidate genes (involvement in DNA damage response, proliferation, apoptosis, cell mobility, stress response, etc.) were also taken into account. In addition, interactions with known tumor suppressor genes and oncogenes were analyzed using BioGrid and String databases as well as by WebGestalt functional enrichment analysis (see Web Resources for additional information). We also considered mutations whose relationship with cancer has been already mentioned in the scientific literature. These efforts permitted us to compose a list of selected candidates, which included 229 variants (Fig. 2, Supplementary Table S4).

Eighty-four top candidates were subjected to validation by Sanger sequencing of index DNA samples. The presence of the variant was confirmed for 79 (94.0%) samples. Pilot case–control study involving high-risk BC patients and healthy middle-aged women allowed to classify the analyzed variants for three categories (Supplementary Fig. S1, Supplementary Table S5). 39 alleles, although being present in index cases, were not detected in the studied group of cancer patients. Therefore, the disease-predisposing significance of these presumably "private" mutations could not be evaluated within the reasonably powered case–control study. 29 variants demonstrated an apparently similar distribution in high-risk BC cases and controls. Finally, ten alleles were over-represented in BC patients, and therefore, were subjected to extended case–control analysis.

In the second stage of the study, BC-predisposing role was confirmed for six alleles: *USP39* c.*208G > C, *PZP* p.Arg680Ter, *LEPREL1* p.Pro636Ser, *SLIT3* p.Arg154Cys, *CREB3* p.Lys157Glu, and *ING1* p.Pro319Leu. All these variants were detected in the heterozygous state. The description of the above genes and mutations is provided in Table 1 and Supplementary Table S6. LOH analysis revealed only one instance of the loss of the wild-type allele indicating that the somatic deletion of the remaining gene copy is not a key mechanism of BC pathogenesis if driven by these genes. We further pooled together all available BC cases and considered the distribution of the above germline variants in BC subgroups according to the presence of clinical signs of hereditary disease (family history, early-onset, presence of multiple cancers) (Table 2). Statistical significance was achieved for *USP39* c.*208G > C which was strongly associated with triple-negative breast tumors ($p=0.0001$), and for *PZP* p.Arg680Ter and *SLIT3* p.Arg154Cys mutations which tended to associate with the presence of multiple cancers in the studied patients ($p=0.039$ and 0.022, respectively).

We chose the truncating variant in *PZP* (p.Arg680Ter, rs145240281) and the putative splice variant in *USP39* (c.*208G > C in isoform 1, c.*46-1G > C in isoform 2, rs112653307) for further replication in the three independent case–control series. The results from the replication study are provided in Table 3. Both variants were identified in all three populations at heterozygote frequencies between 0.5–1.5%. There was no indication for an association of *PZP* p.Arg680Ter (rs145240281) with breast cancer risk in any of the three studies nor in the combined analysis (OR 0.87, 95% CI 0.52–1.47, $p=0.61$). In case of the *USP39* variant rs112653307, we observed an increased effect size across studies and a nominally significant association with breast cancer risk in the combined analysis (OR 1.72, 95%

Table 1 Prevalence of *USP39* c.*208G > C, *PZP* p.Arg680Ter, *LEPREL1* p.Pro636Ser, *ING1* p.Pro319Leu, *SLIT3* p.Arg154Cys, and *CREB3* p.Lys157Glu alleles in high-risk and consecutive breast cancer (BC) patients

Gene name, mutation	High-risk BC OR (95% CI) *p* value*	Consecutive BC OR (95% CI)	Controls (%)	Somatic loss of the remaining allele in the tumor
USP39 *c.*208G > C*	6/792 (0.75%) 17.6 [1.00–312.60] $p = 0.050$	9/1340 (0.67%) 15.2 [0.89–261.79] $p = 0.060$	0/1066 (0)	0/5
PZP *p.Arg680Ter*	3/792 (0.38%) 4.12 [0.426–39.553] $p = 0.221$	8/1504 (0.62%) 5.78 [0.721–46.246] $p = 0.068$	1/1081 (0.09)	0/4
LEPREL1 *p.Pro636Ser*	2/797 (0.24%) 6.70 [0.321–139.828] $p = 0.220$	6/1500 (0.44%) 9.28 [0.522–164.862] $p = 0.150$	0/1066 (0)	1/4
SLIT3 *p.Arg154Cys*	10/784 (1.28%) 4.40 [1.206–16.036] $p = 0.025$	13/1220 (1.07%) 3.66 [1.041–12.886] $p = 0.043$	3/1023 (0.29)	0/7
CREB3 *p.Lys157Glu*	6/791 (0.76%) 7.62 [0.916 to 63.447] $p = 0.060$	2/1224 (0.16%) 1.65 [0.149 to 18.257] $p = 0.681$	1/1011 (0.1)	0/2
ING1 *p.Pro319Leu*	2/588 (0.34%) 8.91 [0.427–185.976] $p = 0.158$	1/1200 (0.08%) 2.61 [0.106–64.260] $p = 0.556$	0/1045 (0)	0/2

**p*-values for the odds ratio (OR) significance were calculated according to Sheskin, 2004 [60]. Online calculator: https://www.medcalc.org/calc/odds_ratio.php

Table 2 Distribution of newly identified cancer-predisposing alleles in breast cancer (BC) patients depending on the presence of clinical signs of hereditary disease

Gene name, mutation	Family history of breast or ovarian cancer		Early-onset (≤ 50 years)		Multiple cancers		Presence of any clinical sign of hereditary BC (family history or early-onset or multiple primaries)		Triple-negative breast cancer	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
USP39 c.*208G > C	2/314 (0.64%)	13/1818 (0.72%)	8/974 (0.82%)	7/1158 (0.60%)	0/158 (0%)	15/1974 (0.76%)	9/1228 (0.7%)	9/904 (0.7%)	11/264 (4.2%)	3/1159 (0.26%)
PZP p.Arg680Ter	1/331 (0.30%)	10/1965 (0.51%)	6/1046 (0.57%)	5/1250 (0.4%)	3/165 (1.83%)	8/2131 (0.38%)	9/1317 (0.68%)	2/979 (0.20%)	3/266 (1.1%)	6/1167 (0.5%)
LEPREL1 p.Pro636Ser	0/330 (0%)	8/1967 (0.41%)	4/1048 (0.38%)	4/1249 (0.32%)	0/165 0%	8/2132 0.38%	4/1319 (0.30%)	4/978 (0.41%)	0/266 (0%)	8/1167 (0.7%)
SLIT3 p.Arg154Cys	2/302 (0.66%)	21/1702 (1.23%)	12/950 (1.26%)	11/1054 (1.04%)	5/145 (3.45%)	18/1859 (0.97%)	17/1189 (1.43%)	6/815 (0.74%)	5/223 (2.2%)	13/1004 (1.3%)
CREB3 p.Lys157Glu	3/304 (1.00%)	5/1711 (0.29%)	5/958 (0.52%)	3/1057 (0.28%)	1/149 (0.67%)	7/1866 (0.38%)	7/1200 (0.58%)	1/815 (0.12%)	1/223 (0.4%)	6/1010 (0.6%)

ING1 p.Pro319Leu was not included in the analysis due to low frequency of mutations. Triple-negative BC was significantly associated with the presence of *USP39* c.*208G > C mutation ($p = 0.0001$). Multiple cancers occurred statistically more often in *PZP* p.Arg680Ter and *SLIT3* p.Arg154Cys mutation carriers ($p = 0.039$ and 0.022, respectively). *PZP*-mutated patients diagnosed with multiple cancers were: BC + colorectal cancer; BC + basal cell carcinoma; bilateral BC + gastric cancer; all *SLIT3*-mutated cases with multiple cancers represented bilateral BC. Other comparisons between BC subgroups produced p-values below the statistical significance threshold

Table 3 Replication study for the *PZP* rs145240281 and *USP39* rs112653307 variants

Gene	Variant	Study	Country	BC cases	Controls	OR (95% CI)	*p*
USP39	rs112653307 c.*208G > C	HMBCS	Belarus	20/1864 (1.07%)	8/1214 (0.66%)	1.64 [0.72–3.72]	0.24
		HUBCS	Russia	5/446 (1.12%)	2/417 (0.48%)	2.35 [0.45–12.19]	0.31
		HaBCS	Germany	22/906 (2.43%)	13/890 (1.46%)	1.68 [0.84–3.35]	0.14
		Combined		47/3216 (1.46%)	23/2521 (0.91%)	1.72 [1.04–2.84]	0.04
PZP	rs145240281 p.Arg680Ter	HMBCS	Belarus	20/1862 (1.07%)	16/1214 (1.32%)`	0.81 [0.42–1.57]	0.54
		HUBCS	Russia	2/447 (0.45%)	2/420 (0.48%)	0.94 [0.13–6.70]	0.95
		HaBCS	Germany	6/906 (0.66%)	9/891 (1.01%)	0.65 [0.23–1.84]	0.42
		Combined		28/3215 (0.87%)	27/2525 (1.07%)	0.87 [0.52–1.47]	0.61

Genotyping results for the *PZP* rs145240281 and *USP39* rs112653307 variants in the Hannover–Minsk Breast Cancer Study (HMBCS), the Hannover–Ufa Breast Cancer Study (HUBCS), and the Hannover Breast Cancer Study (HaBCS). For HUBCS, only individuals with documented Russian ancestry were included. Cases and Controls are given as numbers of heterozygotes versus total number

OR odds ratio; *CI* confidence interval

OR and *p* are the values from logistic regression analyses for the single studies and from a fixed-effects meta-analysis in the combine

CI 1.04–2.84, $p = 0.035$) that was consistent with the data obtained in the St.-Petersburg cohorts.

We also analyzed the presence of 6 candidate BC-predisposing variants (*USP39* c.*208G > C, *PZP* p.Arg680Ter, *LEPREL1* p.Pro636Ser, *SLIT3* p.Arg154Cys, *CREB3* p.Lys157Glu, and *ING1* p.Pro319Leu) in 21 patients who carried germline mutations in known hereditary cancer genes (Supplementary Table S2). No instances of co-occurrence of known and novel BC-associated alleles were observed.

Discussion

This study revealed a possible contribution of six novel BC-predisposing genetic variants to the burden of genetic breast cancer risk in Russia. Two of these alleles (*USP39* c.*208G > C and *PZP* p.Arg680Ter) had been classified as a protein-truncating or splice site variant, respectively, while the remaining ones are missense mutations with high CADD score.

USP39 (ubiquitin specific peptidase 39, or snRNP assembly defective 1 homolog) plays a role in pre-mRNA splicing as a component of the spliceosome; it also maintains the spindle checkpoint and supports successful cytokinesis. Upregulation of USP39 has been associated with stimulation of cancer cell proliferation in vitro and in vivo [28]. *USP39* knockdown inhibits cell proliferation and colony formation in breast, gastric, hepatocellular, etc. cancer cell lines and induces apoptosis in tumor cells [29–32]. Interestingly, the USP39 deubiquitinase has recently been identified as an upstream regulator of the checkpoint kinase 2, CHEK2, which plays a well-known role in breast cancer susceptibility. Knockdown of USP39 led to deregulated CHEK2, compromising the DNA damage-induced G2/M checkpoint, decreasing apoptosis, and conferring cancer cells resistance to chemotherapy drugs and radiation treatment [33]. *USP39* c.*208G > C (rs112653307) is a splice-acceptor variant, which is likely to alter the splicing of the last exon, as the usage of this acceptor site has been documented for the mRNA isoform 2 of *USP39*. It is thus possible that it affects the processing of the corresponding 3'UTR. The 3'UTR of the *USP39* gene harbors a number of regulatory elements, most notably, the target site for tumor suppressor *miR-133a* [34–36]. In particular, *miR-133a* suppresses cell proliferation by targeting *USP39* and predicts better prognosis in gastric and pancreatic cancer [37, 38]. Thus, the c.*208G > C (rs112653307) variant could disrupt the *USP39* gene splicing and prevent its downregulation by *miR-133a*. While this variant was not found in over 1000 Russian controls of stage 1, it affected 0.7% of Russian breast cancer cases; moreover, it was strongly associated with triple-negative breast tumors. Interestingly, its association was replicated in a combined analysis of three additional populations, although the effect size was found to be modest in these

population-based studies. From our data, *USP39* represents an interesting and novel candidate breast cancer susceptibility gene.

PZP gene encodes for the so-called pregnancy zone protein which serves as an inhibitor of proteinases [39, 40]. Recently *PZP* has been described as a novel biomarker for predicting the prognosis of hepatocellular carcinoma [41]. The same loss-of-function variant (*PZP* p.Arg680Ter) has been previously identified by whole exome sequencing of Brazilian patients with clinical signs of hereditary breast cancer [42] and in a multicase breast cancer family [43]; no segregation data was provided in the latter report. However, our replication study suggests that the role of *PZP* p.Arg680Ter for breast cancer risk, if any, is limited and excludes more than 1.5-fold risks.

The four missense variants also target genes with potentially relevant function in cancer development. *LEPREL1* (leprecan-like 1 protein) also known as *P3H2* (prolyl 3-hydroxylase 2) is involved in the post-translational modification of collagen type IV. It was shown to inhibit proliferation of cancer cells [44]. Methylation of this gene is frequently observed in estrogen receptor-positive breast carcinomas [45]. *SLIT3* (Slit Guidance Ligand 3), also known as *MEGF5* (Multiple EGF-Like Domains Protein 5), has a tumor suppressor role [46, 47]. In particular, it was shown to inhibit growth of mammary carcinomas in mice [48]. Low expression of SLIT3 is associated with decreased sensitivity of hepatocellular carcinoma cells to cytotoxic therapy [49]. *CREB3* (cAMP responsive element binding protein 3) regulates cell proliferation and migration as well as plays a role in tumor suppression [50]. One of *CREB3* protein isoforms is able to inhibit estrogen receptor alpha-mediated signaling leading to suppression of cell division in breast cancer [51]. *ING1* (inhibitor of growth family, member 1) gene may induce growth arrest, cell senescence, and apoptosis [52, 53]. Loss of *ING1* expression is characteristic for a broad range of cancer types [54], and decreased level of *ING1* gene product is associated with higher rate of metastases in breast cancer patients [55, 56]. Although all four variants have been classified as potentially pathogenic by SNPEff, more work would be needed to determine the functional relevance of these missense substitutions.

None of the analyzed variants demonstrated frequent involvement of the somatic loss of the remaining gene copy in BC pathogenesis. LOH of the wild-type allele is highly characteristic for *BRCA1* and *BRCA2*-driven cancers [57]; however, it is uncommon for BC arising in *CHEK2, NBS1,* and *BLM* mutation carriers [22]. It is of potential interest that *PZP* p.Arg680Ter and *SLIT3* p.Arg154Cys mutations occurred at relatively high frequency in patients with multiple malignancies (3/165 (1.83%) and 5/145 (3.45%), respectively).

Unfortunately, we did not have access to DNA samples obtained from the affected relatives of BC patients who was found to carry presumably BC-predisposing germline mutations. Lack of the genetic segregation data is a weakness in the current report. We performed a systematic analysis of all published family-based WES studies; however, none of them contained relevant information on the BC-associated inheritance of the candidate alleles identified within this study (Supplementary Fig. S2).

The newly identified candidate variants, if confirmed by others, will contribute only to a minor fraction of hereditary BC. That corresponds well with other available BC exome sequencing studies [58, 59]. Our study also has revealed rare deleterious variants in many additional candidate genes but the subsequent screening indicated that they were too rare for a variant-focused association study, and large-scale targeted sequencing studies will be needed to resolve the role of these candidate genes for breast cancer through gene-based association analyses. It is likely that larger exome sequencing approaches might further be fruitful to clarify some of the remaining genetic burden, although there are limitations of this approach as previously discussed [16].

In conclusion, this study suggests six novel genetic variants, which are likely to contribute to BC predisposition. A variant of the CHEK2 regulator USP39 was replicated in additional populations. Functional studies are required to provide biological explanation for the observed gene-disease associations. It is also of notice that only 36.7% of the 229 candidate alleles selected upon exome analysis were subjected to case–control validation in this report. The epidemiological analysis of the remaining 145 mutations is currently underway and is likely to reveal some additional breast cancer susceptibility candidate genes.

Web Resources

- 1000 Genomes Project, http://www.1000genomes.org/
- ANNOVAR, http://annovar.openbioinformatics.org/
- BioGrid, https://thebiogrid.org/
- ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
- CADD, https://cadd.gs.washington.edu/
- COSMIC, https://cancer.sanger.ac.uk/cosmic/
- dbSNP, http://www.ncbi.nlm.nih.gov/SNP
- Ensembl, http://www.ensembl.org/
- Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org
- GeneCards, https://www.genecards.org/
- GNOMAD, http://gnomad.broadinstitute.org/
- HGMD, http://www.hgmd.org/
- IGV browser, http://www.broadinstitute.org/igv/home
- InterVar, https://github.com/WGLab/InterVar/
- MedGen, https://www.ncbi.nlm.nih.gov/medgen/

- Exome Variant Server (ESP), http://evs.gs.washington.edu/EVS/
- RefSeq, http://www.ncbi.nlm.nih.gov/refseq/
- String, https://string-db.org/
- UniProtKB, https://www.uniprot.org/help/uniprotkb
- WebGestalt, http://www.webgestalt.org/

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study design was approved by the local Ethical Committee. All procedures performed in study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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