RESEARCH ARTICLE

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Pathogenicity Reclassification of Genetic Variants Related to Early-Onset Breast Cancer among Women of Mongoloid Origin

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Abstract

Background: Germline alterations in *BRCA1, BRCA2*, and other genes are responsible for early-onset breast cancer. However, up to 20% of molecular tests report genetic variant of unknown significance (VUS) or novel variants that have never been previously described and their clinical significance are unknown. This study aimed to reclassify variant of unknown significance (VUS) or novel variants by using the ActiveDriveDB database that annotates variants through the lens of sites of post-translational modifications (PTM). **Methods:** Our study included thirty-eighth young Buryat BC patients, belonging to the Mongoloid race and anthropologically to the Central Asia. Genomic DNA was extracted from the peripheral blood lymphocytes using the phenol/chloroform method. DNA library were prepared using the Hereditary Cancer SolutionTM kit (Sophia GENETICS, Switzerland) to cover 27 genes, such as *ATM, APC, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, EPCAM, FAM175A, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PIK3CA, PMS2, PMS2CL, PTEN, RAD50, RAD51C, RAD51D, STK11, TP53*, and *XRCC2*. Paired-end sequencing (2 x 150 bp) was conducted using NextSeq 500 system (Illumina, USA). **Results:** We re-examined 135 rare variants (41 VUS, 25 conflicting, 64 benign and 5 new variants). We identified 10 out of 135 (7.4%) mutations that affected the sites of post-translational modification in proteins. Of 135 rare mutations, 1 benign variant was reclassified as network-rewiring - motif loss mutation, 3 VUS and 1 new variant were reclassified as distal PTM- mutations, 2 new and 1 benign variant were classified as proximal PTM- mutations and 1 benign and 1 conflicting variant were classified as direct PTM- mutations.**Conclusions:** For the first time, 7.4% (10 out of 135) of mutations that affected the sites of post-translational modification in proteins were identified among early-onset breast cancer women of Mongoloid origin.

Keywords: VUS- germline mutation- breast cancer- Mongoloid race- Central Asia

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Introduction

Breast cancer (BC) is the most common female malignancy worldwide. Germline alterations in homologous recombination repair genes (*ATM, APC, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2* and other) are responsible for early-onset BC or ovarian cancer incidence in 7-15% patients among different ethnic groups. Individuals with clinically significant inherited mutations should be offered the risk-reduction strategies, surveillance and chemoprevention (Dutil et al., 2019). Currently, the molecular diagnosis of hereditary breast and ovarian cancers has become more available with the use of next-generation sequencing. Next generation sequencing allows detecting DNA damage of five levels of pathogenicity (not pathogenic, likely not pathogenic, uncertain, likely benign, benign). However, up to 20% of molecular tests report genetic variant of unknown significance (VUS) or novel variants that have never been previously described and their clinical significance are unknown. The interpretation of VUS is still a major challenge for specialists involved in NGS data analysis and therapy administration. Misinterpretation of VUS can lead to serious clinical mistakes for patients and their families. In accordance to literature data, Asians and African-Americans have higher rates of VUS then well-characterized Caucasians (Park et al., 2017). An existing open access databases (ClinVar, BIC, and

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ENIGMA and other) play an important role in the interpretation of VUS, but in Asian populations the interpretation of VUS is still difficult due to restricted data.

Breast cancer related gene aberrations are known to be various in different ethnic groups. There are limited data on hereditary BC associated mutations in Buryat BC patients (small nationality of Russia, up to 0,5%). Republic of Buryatia is a federal subject of Russia located in Central Asia or in the south of Eastern Siberia. Buryat, belonging to the Mongoloid race and anthropologically to the Central Asia. Our previous study has been aimed to identify the BC-associated genes in 38 Russian Mongoloid BC patients living in Russian Siberia (Buryats). The pathogenic variants in two non-BRCA1/2 susceptibility genes, such as RAD51D (rs137886232) and PTEN (rs786201044) were found only in three BC patients aged under 45 years old (Gervas et al., 2020). Thus, 8% (3/39) of patients harbored one pathogenic variant and 92% of patients with early-onset BC had rare VUS, conflicting and benign variants. Thus, there is a growing need for scientifically rigorous additional approaches for extended annotation of rare variants.

The open-source database is available at https://www.ActiveDriverDB.org. The human proteo-genomics ActiveDriveDB database contains more than 385,000 mutations associated with post-translational modification sites (*PTM* mutations) and numerous amino acid substitutions from The Cancer Genome Atlas, ClinVar and other projects. This program simulates site-specific interaction networks of proteins with upstream enzymes and approved drugs (Krassowski et al., 2018). This study aimed to reclassify variant of unknown significance (VUS) or novel variants by using the ActiveDriveDB database that annotates variants through the lens of sites of post-translational modifications.

Materials and Methods

Patients recruitment was carried out during the expedition on the territory of Republic of Buryatia, namely in the GBUZ "Buryat Republican clinical oncology dispensary". Information, including age at diagnosis, family history and origin was obtained by self-reported questionnaire.

Blood was collected in blood tubes containing K2EDTA. Genomic DNA was extracted from the peripheral blood lymphocytes using the phenol/chloroform method. Purity of the DNA was determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and then quantified by Qubit 2.0 fluorometer and HS dsDNA Assay Kit (Thermo Fisher Scientific, USA). Integrity of the DNA (DIN) was verified on a 2200 TapeStation system (Agilent, USA). DNA library were prepared using the Hereditary Cancer SolutionTM kit (Sophia GENETICS, Switzerland) to cover 27 genes, such as *ATM, APC, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, EPCAM, FAM175A, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PIK3CA, PMS2, PMS2CL, PTEN, RAD50, RAD51C, RAD51D, STK11, TP53*, and *XRCC2*. Paired-end sequencing (2 x 150 bp) was conducted using NextSeq 500 system (Illumina, USA).

Bioinformatics analysis

Sequencing data was analyzed according to the GATK (Genome Analysis Toolkit) best practice recommendation for Whole Exome Sequencing using GRCh37 as a reference for Burrows-Wheeler alignment. The obtained variants were annotated with ANNOVAR software and ranged according to population frequency (genomic exome, gnomAD genome, and ExAC), ClinVar, CADD, and literature data (McKenna et al., 2010; DePristo et al., 2011; Van der Auwera et al., 2013;). Detected sequence variants were annotated using PolyPhen2, Mutation Taster, and SIFT (Kumar et al., 2009; Adzhubei et al., 2010; Schwarz et al., 2014).

The open-source database is available at https://www.ActiveDriverDB.org (Ontario Institute for Cancer Research). The VCF files were aligned to hg19/GRCh37 version of the human genome. NGS data were further annotated regarding their position in post-translational modifications sites. Depending on the location of amino acid substitutions in the post-translational modification sites, mutations can be described as direct (central amino acid residue of the PTM site) or indirect (proximal or distal if they replace 1–2 or 3–7 amino acid residues from the nearest PTM site, respectively). An assessment of the network impact of mutations also was carried out using ActiveDriverDB.

Results

Our previous study aimed to identify the BC-associated genes in 38 Russian Mongoloid BC patients (Buryats) by NGS. The median age of patients at BC diagnosis was 42 years (range: 26 - 55). Eighty-one percent of patients were diagnosed with BC before the age of 50. More than one-third of patients under the age of 50 had a family history of BC. Almost all tested women were diagnosed with invasive (ductal) carcinoma of no special type. All tested women were previously assessed as negative for *BRCA1/2* mutation (*BRCA1 5382insC, BRCA1 185delAG, BRCA1 4153delAG, BRCA1 T300G, BRCA2 6174delT*) which consider as pathogenic in Slavic population.

In this study we re-examined 135 rare variants, including 41 variants reported to be VUS, 26 conflicting variants, 64 benign/likely benign variants, and 5 new variants. We identified 10 out of 135 (7.4%) mutations that affected the sites of post-translational modification in proteins. Rare genetic variants classified by ActiveDriverDB as PTM-associated mutations are given in Table 1 and Figure 1.

Distal PTM-associated mutations of *MLH1* gene (rs4986984) were found in two young BC patients with no burdened family history. Direct PTM-associated mutations of *EPCAM* gene (rs74531854) were found in three BC patients with burdened family history and in one young patient with no burdened family history. Another network-rewiring - motif loss mutations of *NBN* gene (rs192236678) were found in a 40-year-old patient with no burdened family history (Figure 2).

Proximal PTM-associated mutations of *BRCA1* gene (17:41244039:A>G) were found in a 45-year-old BC patient. ActiveDriverDB analysis suggests that

Figure 1. a – c: Zoomed Needleplot Shows Germline Disease Mutations Located in Phosphorylation Sites. Only PTM-associated mutations are shown

Table 1. Rare Genetic Variants Classified by ActiveDriverDB as PTM-Associated Mutations

CLNSIG dbPubMed	Gene	dbSNP ID	Chr: position:substitution (MAF)	SIFT/PolyPen2	PTM-associated mutations localization	BC patients age	Family history
Benign /Likely benign	*BRCA2*	rs28897727	13:32912750:G>T T=0.006497 (GnomAD_exome)	tolerated/ probably damaging	Proximal	36	Grandmother - stomach cancer
	EPCAM	rs74531854	2:47604176:C>T ,T=0.005685 (GnomAD_exome)	deleterious/ benign	Direct	48	No data
						54	Sister – breast cancer
						55	Sister - adrenal cancer
						36	Grandmother - stomach cancer
	NBN	rs192236678	8:90965508:G>T T=0.0003 (GnomAD_exome)	tolerated/ benign	Network-rewiring - motif loss	40	No burdened family history
VUS	*MLH1*	rs4986984	3:37053562:C>T T=0.0003 (ExAC)	deleterious/ probably damaging	Distal	46	No burdened family history
						26	No burdened family history
	MSH6	rs61756469	2:48010479:C>T T=0.0001 (ExAC)	Tolerated low confidence/ benign	Distal	32	No burdened family history
	BRCA2	rs80359254	13:32972584:A>G G=0.0000 (ExAC)	tolerated/ benign	Distal	49	No burdened family history
Conflicting	*ATM*	rs150757822	11:108183194:A>C C=0.0003 (ExAC)	tolerated/ benign	Direct	49	Sister - cervical cancer
New mutation	*APC*	-	5:112178383:G>A	-	Distal	55	Sister - adrenal cancer
	BRCA1	-	17:41244039:A>G	-	Proximal	45	No data
	MSH2	-	2:47705411:G>C	-	Proximal	47	No data

17:41244039:A>G substitution of BRCA1 gene may induce gains of phosphosites of the PLK1 kinase that pharmaceutically targetable by Fostamatinib (Figure 3).

Discussion

Variants of uncertain significance (VUS) or unclassified variants (UVs) are rare missense substitutions, including in-frame deletions or insertions, silent coding alterations, intronic changes. Variants of uncertain significance have unknown functional effects on proteins and cannot be classified as either «Pathogenic» or «Not Pathogenic». The frequency of VUS for BRCA1/2 genes accounts for 30% - 50% in many countries. In the USA, these variants account for 5% - 10% due to ongoing classification efforts (Hofstra et al., 2008). The interpretation of VUS is a major challenge for specialists involved in NGS data analysis and therapy administration. For example, open-access databases and research consortia (ClinVar, the BIC, ENIGMA and other) playing an important role for the interpretation of VUS for patients from Western countries are not suitable for variants found in Asian or other ethnic populations (Tsai et al., 2019). Moreover, the authors suggest that caution should be exercised when analyzing data of Asian populations (Chinese, Japanese, Koreans). For example, the use of ExAC EAS (East Asian), which is mainly composed of Chinese and Japanese, led to misleadingly in assessing the frequency of variants found among Koreans. It became apparent when an extended own control group consisting of Korean population was used. Tsai (2019) reported that family studies are an important source for classification of rare VUS in the general population. There is a FindMyVariant.org website designed to educate and assist individuals in pursuing family studies for VUS reclassification. At the same time, the authors note that VUS reclassification through family studies has a number of limitations (communicating with families, collecting family history for building pedigrees, interest and awareness of patients). Pål Møller (2007) reported that family history detected less than 50% of the mutation carriers (Møller et al., 2007).

In our study, along with the well-known ClinVar, PolyPhen2, Mutation Taster, and SIFT databases we used the open-source database https://www.ActiveDriverDB.

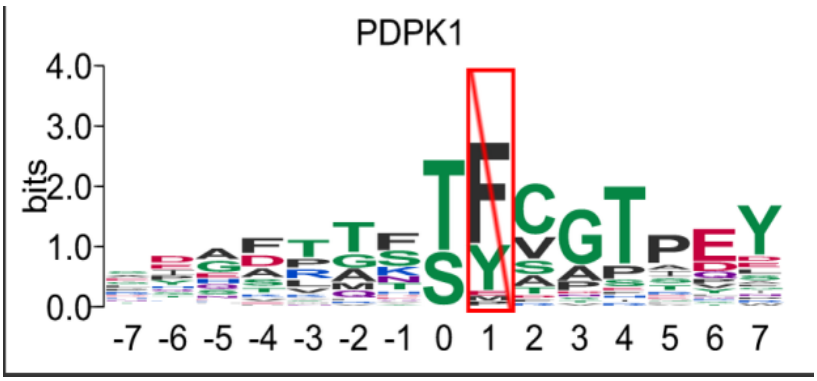

Figure 2. Zoomed Needleplot Shows Germline Disease Mutations Located in Phosphorylation Sites of NBN Gene. The mutation rs192236678 of NBN gene is predicted to disrupt the sequence motif of the PDPK1 kinase

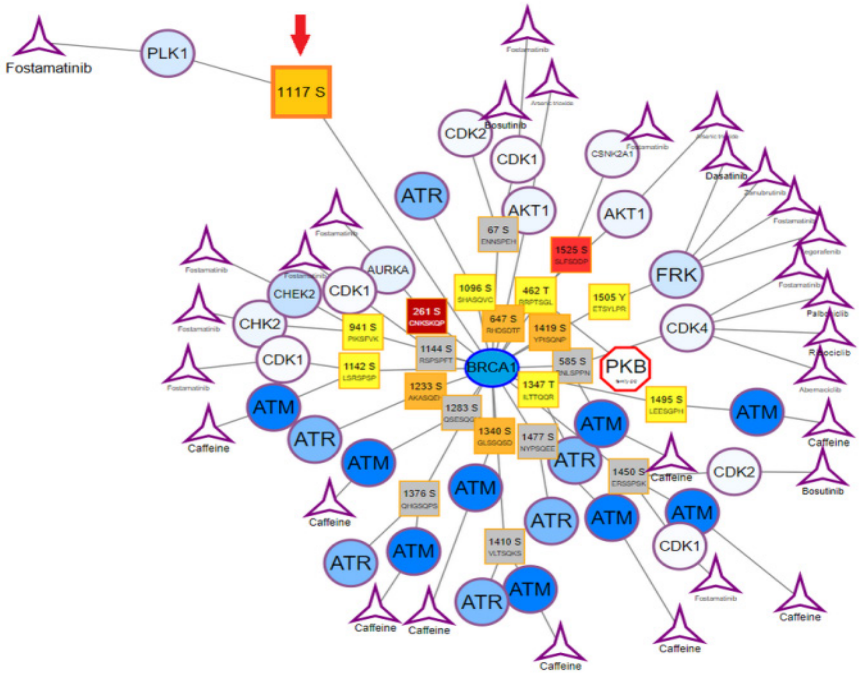

Figure 3. The Computationally Derived PTM Interaction Network of BRCA1, Kinases Predicted to Interact with Mutant BRCA1, and Drugs Targeting the Kinases. Arrows point to the mutations 1117S.

org (Ontario Institute for Cancer Research), which annotates mutations through the prism of post-translational modification sites (PTMs). After translation, proteins undergo different post-translational modifications (PTMs) or chemical modifications (phosphorylation, glycosylation, ubiquitination, SUMOylation, acetylation, and lipidation) that impact their activity and function. PTMs influence many processes in the cell, including protein activation and degradation, protein-protein interactions, chromatin organization, development, and signaling pathways associated with different types of cancers (Samaržija et al., 2021). Mutations at PTM sites could alter sequence motifs linked by PTM enzymes and can affect signaling networks. Further, PTMs provide potential sites of intervention and could be used in precision cancer therapies. Krassowski (2018) reported that up to 30% of mutations in PTM sites were considered «benign» by tools such as PolyPhen2, SIFT and others. ActiveDriverDB were designed as proteo-genomics resource to find mutations located in the human PTM sites mutations, to display the network context, to visualize protein-protein interactions and to apply the drugs targeting PTM enzymes.

Network-rewiring - motif loss mutations of *NBN* gene. Nibrin, encoded by the NBN gene recognize the DNA double-strand breaks during the non-homologous end- joining (NHEJ) and induce cell-cycle checkpoint to provide genomic stability. Mutations in the *NBN* gene lead to Nijemen breakage syndrome, which may result in particularly susceptibility to cancer, including breast cancer (Uzunoglu et al., 2016). In our study, network-rewiring - motif loss mutation of *NBN* gene (rs192236678) was found in a 40-year-old BC patient with no burdened family history. The rs192236678 mutation of NBN gene is predicted to disrupt the sequence motif and change the identity of these residues and specificity the PDPK1 kinase. It is well known that 3-phosphoinositide dependent protein kinase 1 (PDPK1 kinase) is the first goal in the PI3K/AKT/mTOR pathway signaling. The oncogenic potential of aberrant PI3K pathway signaling through PDK1 to AKT has been well demonstrated (Maurer et al., 2009). Given that the significance of the affected phosphorylation sites is well known, these network-rewiring - motif loss mutations of *NBN* gene could be considered as candidates for further studies.

New PTM-associated mutations of *BRCA1* gene associated with drugs targeting kinase. BRCA1 plays a crucial role in DNA repair, cell cycle control and genomic stability (Wang et al., 2009). Germline *BRCA1* mutations are the main hallmark for hereditary breast and ovarian cancers. Proximal PTM-associated mutation of *BRCA1* gene (17:41244039:A>G) was found in a 45-year-old BC patient. ActiveDriverDB analysis suggests that 17:41244039:A>G substitution of *BRCA1* gene may induce gains of phosphosites of the PLK1 kinase that is pharmaceutically targeted by Fostamatinib. PLK1 kinase is a critical regulator of the cell cycle and an inhibitor of apoptosis. PLK1 kinase overexpression in various tumors has been associated with poor prognosis. Although a number of small molecule inhibitors of PLK1 have been studied as anticancer agents (Abramson, 2016). Shinde (2019) in vivo data suggest that Fostamatinib could be considered as an effective treatment option for the prevention of metastatic recurrence in breast cancer.

Rare variant of *EPCAM* gene impacting the phosphorylation site. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein mediating Ca^{2+}-independent homotypic cell–cell adhesion in epithelia. EpCAM is involved in cell signaling, migration, proliferation, and differentiation. Deletions in EPCAM

gene are associated with Lynch hereditary cancer syndrome leading to different types of cancer (Morris et al., 2016). Direct PTM-associated rare mutation of *EPCAM* gene (rs74531854, 2:47604176:C>T, T=0.005685 (GnomAD_exome)) was found in three BC patients with burdened family history and in one young patient with no burdened family history. The significance of the rs74531854 of the EPCAM gene may be revised in the future, taking into account its frequency, location in the center of the phosphorylation site, as well as the fact of its detection in three BC patients with a family history.

Our study included 38 Buryat BC patients, belonging to the Mongoloid race and anthropologically to the Central Asia. Republic of Buryatia is a federal subject of Russia located in Central Asia or in the south of Eastern Siberia (Cherdyntseva et al., 2017). Buryats are characterized by molecular diversity due to the long generation time or the mixed nature of origin compared with other ethnic groups living in Siberia (Stepanov et al., 2003; Stepanov et al., 2003; Khitrinskaia et al., 2010; Har'kov et al., 2014). It is obvious that a more detailed genetic analysis of the Buryats is required.

Due to the fact that the territory of Eastern Siberia remains unaffected by large-scale studies, mutations in *BRCA1/2* genes associated with BC have not yet been found in Buryats. However, the cancer burden in Buryats has risen and the cancer risk assessment has been limited (Cherdyntseva et al., 2014). Modern approaches of molecular oncology have a sufficient depth to solve issues of early diagnosis of hereditary BC in ethnic groups. ActiveDriverDB is a great alternative approach for annotation of genetic variants found in Buryats population. The ability to assess the effects of specific genetic variants in a biological context makes ActiveDriverDB uniquely informative for reclassification of VUS and other findings.

Our data are consistent with other studies in the field of VUS reclassification of *BRCA1/2* genes. The problem regarding VUS reclassification has been discussed mostly for Asian populations, where the frequency of VUS reaches 60% (Nakamura et al., 2016; Kwong et al., 2016; Chian et al., 2021). In the study of Sinha (2020), the authors created own model where they measured the effect of variants on the structural conformations of BRCT repeats using molecular dynamics simulation (MDS) consisting of RMSD (Root-mean-square-deviation), RMSF (Root-mean-square-fluctuations), Rg (Radius of gyration), SASA (Solvent accessible surface area), NH bond (hydrogen bond) and Covariance analysis. Using this approach, were analyzed 131 variants, consisting of 89 VUS (variant of undetermined value) and 42 unclassified variants (unclassifiable by current methods) within the BRCT repeats, and were able to differentiate them into 78 deleterious and 53 tolerated variants (Sinha et al., 2020). Verónica Castillo-Guardiola (2022) applied an algorithm to prioritize VUS, and out of the 70 VUS, 19 were classified as variants with high-risk of having deleterious effect that needed to be further explored (Castillo-Guardiola et al., 2022). Thus, the ActiveDriverDB tool, own models or algorithms are great alternative approach for genetic variants annotation.

In conclusion, in our study, out of 135 rare mutations, 10 were reclassified as PTM-associated mutations (7.4%). Given the fact that the population of Buryats is poorly studied, the data obtained using ActiveDriverDB cannot be neglected. We reclassified not only rare VUS and benign mutations, but also newly found mutations. It is obvious that a more detailed genetic analysis of our findings in Buryat population is required to identify pathogenic mutations and for effective patient monitoring.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Choynzonov, N. Cherdyntseva, L. Pisareva; data collection: A. Molokov, E. Yumov; analysis and interpretation of results: P. Gervas, A. Kiselev, A. Zarubin; draft manuscript preparation: P. Gervas, N. Babyshkina. All authors reviewed the results and approved the final version of the manuscript.

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Ethical Declaration

The current study was approved by the review board of the Cancer Research Institute, and written informed consent was obtained from all patients and in compliance with the recommendations of the Helsinki Declaration.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

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