Short Communications

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Functional Analysis of a Splicing Variant of the *DOCK8* Gene in a Patient with Breast Cancer (Buryat Ethnicity)

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Abstract

Objective: *BRCA1/2* founder mutations have been detected in various populations and ethnicities. However, molecular diagnosis of HBOC remains challenging in populations where founder mutations have not yet been identified. There is limited data on hereditary breast cancer (BC) mutations in the ethnic groups of Siberia. The purpose of this study was to identify new hereditary BC variants in the understudied Buryat ethnic group by using whole exome sequencing (WES) data and DNA constructs for subsequent validation. **Methods:** Our study included a 52-year-old Buryat breast cancer (BC) patient with a family history of BC (her sister was also diagnosed with BC). To identify both novel and previously described variants obtained through whole exome sequencing (WES), we used the OpenCRAVAT mutation impact scoring algorithm, which incorporates a comprehensive knowledge base. For variants requiring further analysis, the DNA construct method was employed. **Result:** According to whole exome sequencing (WES) data, no pathogenic variants were identified in the 52-year-old Buryat breast cancer (BC) patient with a family history of BC. However, the patient carried rare variants of unknown significance: *MLH1* c.550C>T (p.R184C), *FANCI* c.1111A>G (p.S371G), and *DOCK8* c.986C>T (reported as a secondary finding). *In silico* analysis indicated that only the DOCK8 c.986C>T variant may affect splicing. As DOCK8 is a key immunity gene, it could potentially act as a tumor suppressor. Electrophoresis of PCR fragments, obtained using cDNA as a template, showed that in the presence of the c.986C>T variant, the PCR product was 60 bp shorter than the product amplified from the reference sequence. **Conclusion:** We suggest that the combined carriage of the *FANCI* c.1111A>G mutation and the *DOCK8* c.986C>T variant identified in this patient may increase the risk of developing breast cancer (BC). Our functional data indicate a potentional impact of the *DOCK8* c.986C>T variant on splicing. However, the role of this variant in BC pathogenesis and its prevalence within the Buryat ethnic group remain to be elucidated.

Keywords: Splice variant- *DOCK8*- breast cancer- DNA construct- ethnic groups- Buryat non-Caucasian

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Introduction

Breast cancer (BC) ranks as the first most frequently diagnosed cancer among women [1]. Up to 10% of all BC cases and 20% of ovarian cancer (OC) cases are attributed to hereditary breast and ovarian cancer syndrome (HBOC) due to *BRCA1* or *BRCA2* germline mutations. *BRCA1* and *BRCA2* founder mutation (recurrent variants in specific area) have been detected in various populations and ethnicities such as Ashkenazi-Jewish, Polish, Norwegian, Icelandic, and others. It is well established that testing for *BRCA1/2* founder mutations in patients from these ethnic groups is a cost-effective approach and leads to early detection and diagnosis of HBOC [2]. However, molecular diagnosis of HBOC remains challenging for populations where founder mutations have not yet been identified. Indeed, there are limited data on hereditary BC mutations in ethnic groups of Siberia, including the Buryats.

Republic of Buryatia is a federal subject of Russia located in Central Asia or in the south of Eastern Siberia. Buryats are a small Russian ethnic group, with a population estimated at 550,000 people, belonging to the Mongoloid race. Buryats have a significant cancer burden and limited access to genetic cancer risk assessment due

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to the lack of *BRCA1*/2 founder mutations [3]. In this regard, we previously initiated a study to identify the variants in BC associated genes in young Buryat patients living in Siberia using targeted sequencing (n=38). The pathogenic variants in two non-*BRCA1*/2 genes (*RAD51D* and *PTEN*) were found in BC patients aged less than 45 years old (8%, 3/38) [4].

Whole-genome sequencing (WES) technology enables the targeted identification of new causal gene variants in understudied populations, like the Buryats. However, the implications of WES technologies are mainly based on the chance of unsolicited and secondary findings such as variants with a conflicting or unknown significance that can be serving to hypothesis generating [5, 6]. On the other hand, the presence of genetic variants of conflicting significance complicates diagnosis, treatment decisions, prognosis, and disease outcomes [2]. Criteria for assessing the pathogenicity of genetic variants of conflicting significance have been developed, including the guidelines for bioinformatics data analysis, conduction of functional studies (DNA constructs or CRISPR-cas9), etc. Functional studies of genetic variants are an essential method for reclassifying genetic variants of conflicting or unknown significance, thereby shedding light on the etiology of various diseases. For example, DNA constructs are essential tools in genetic engineering, enabling researchers to tailor genetic material for a variety of purposes, from studying gene function to producing therapeutic proteins.

The aim of this study was to search for new genes associated with hereditary BC in the understudied Buryat ethnic groups by using WES. To achieve the goal, all variants leading to hereditary pathology, including congenital immunopathology were taken into account, since it is well known that malignancies are observed more frequently in individuals with primary immunodeficiency. Mutations in immune-related genes, combined with low-penetrance susceptibility gene variants, create a significant genetic burden that increases the risk of developing cancer at an early age [7-9].

Materials and Methods

Initially our study included 35 Buryat patients with histologically confirmed primary breast/ovarian cancer who completed an anonymous questionnaire about basic information. 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 (data not shown). The nationality of the patients was determined using a developed questionnaire. Clinical information was based on the medical report.

WES and data Analysis

We have been using a combined, two-step strategy, based on targeted genes panel as a first NGS screening (data not shown), followed by WES

DNA libraries were prepared using a BGI Optimal DNA Library Prep kit. An Agilent SureSelect Human All Exon V6 kit was used for hybridization. High-throughput sequencing was performed on a DNA nanoball sequencing platform DNBSeq-G400 (depth of coverage is 103.9x, Q30 reflects a base call accuracy of 95%). Exome sequencing data were processed using the DRAGEN Bio-IT platform v.3.9.5 (Illumina) and aligned to the hg38 reference human genome. The quality of sequencing data was controlled using the MultiQC v.1.11 software. All found variants passed the filtering ($p < 0.005$). Therapeutic implications were inferred from OpenCravat a comprehensive knowledge base [10].

Patient Selection

According to WES data, no pathogenic variants were found in patients of the Buryat ethnic group (data not shown). A 52-year-old Buryat BC patient with a family history (sister has BC) was found to have rare variants of unknown significance (*MLH1* c.C550T:p.R184C and FANCI c.A1111G:p.S371G). The c.986C>T *DOCK8* variant was initially considered as secondary findings. Further analysis of the identified variants using predictor programs showed that variants of the *MLH1*, FANCI and *DOCK8* genes potentially linked to disease. In silico analysis indicated that *MLH1* c.C550T:p.R184C and FANCI c.A1111G:p.S371G variants do not affect splicing that limits the study of their clinical significance in vitro experiments (Table 1). To assess the functional significance of c.986C>T variant of the *DOCK8* (p.Ala329Val), an approach using genetically engineered DNA constructs was chosen.

Minigene a small gene construct

For amplification and subsequent cloning in a vector of the genomic fragment containing introns 9 and 10 of exon 9 of the *DOCK8* gene and the flanking intron regions of at least 100 nucleotide pairs (bp) in length, primers with linker sequences of the vector at the ends were selected:

F: 5'-ACCAGAATTCTGGAGCTCGAG GGAACATCTCTCAAATGGCTCC-3
R: 5'-ATTAAGGAGTGTATTAAGCTT GGGGTAAACTGAGAAGGAAACA-3

The minigene insert sequence included a 212bp fragment of intron 8 adjacent to exon 9, exon 9 (150bp), and a 140bp fragment of intron 9. The exon 9 sequence is highlighted in bold and capital letters, the exon region and primer sequences are represented in capital letters in italics, and the intron regions are represented in lowercase letters:

*GGAACATCTCTCAAATGGCTCC*AGAATACCTAGACATTGTGACAAAACATTT
GTCAGATTTATCCAGGAGCCACTTTCCTAAACCACATCTGTGATTATTACA
CTTACTCCTTCAGCAAAATTTCTCTGTGGTGTTTTTACTCCTTTTTAACATGT
TTAAACCATGAATGCAACAGGTCTAACTTATATTTCACTTTGCTGCTCATTT
ACAG**ATCTCAGAAAATTTTCACTGTGACCTGAACTCTGACCAGTTCAA**
AGGATTTCTGCGAGCTCACACGCCTTCAGTGGCCGCATCAAGTCAGGC
GAGATCTGCAGTCTTCTCAGTCACCTACCCGTCCTCAGACATCTACCT
GGTAGTCAAGGTAATTCAGTACGATCTGATTTGCCCAATCTGATGTTTTCA
TTGCTGTGTTGTTCCCAGCACATGTTTGGGGTGCACGCAATCTCAGAATGT
GTCCACATATCCTCTGAGATTCACTT*TGTTTCCTTCTCAGTTTACCCC.*

Using these primers, the specified region was amplified from the patient's genomic DNA using the Q5® High-Fidelity 2X Master Mix kit (New England Biolabs, USA) with the following PCR conditions: first step – denaturation at 98 °C - 30 sec; 2nd step (35 cycles) – denaturation at 98 °C- 10 sec, primer annealing at 60 °C - 15 sec, elongation at 72 °C - 30 sec; 3rd step – final elongation at 72 °C - 2 min.

The used vector pSpl3-Flu2-TKdel was subjected to the action of restriction enzymes XhoI and HindIII (New England Biolabs, USA) to linearize the plasmid. Then, the obtained PCR product and the restricted fragment of the plasmid were purified using the CleanUp kit (Eurogen, Russia). In the next step, the PCR product with a total length of 502 bp was cloned into the vector using the Gibson Assembly® Cloning Kit (New England Biolabs, USA) [11]. The resulting recombinant vectors were transformed into a NEB® 5-alpha Competent *E. coli* cell culture by chemical transformation according to the manufacturer's protocol (New England Biolabs, USA). Colonies containing recombinant vectors were selected by plating the culture on Petri dishes containing solid LB medium with kanamycin (selection marker, 50 μg/ml), followed by subculturing the grown colonies into liquid LB medium. Plasmid DNA was isolated from the overnight NEB® 5-alpha Competent *E. coli* culture. The presence of an insert containing or not containing the *DOCK8*:c.986C>T variant was tested using Sanger sequencing. The transfection experiment with selected plasmids carrying wild-type and mutant inserts was performed in triplicate. Reverse transcription followed by PCR was performed using primers complementary to the exon regions bordering the insert. This step was also performed in triplicate. All resulting fragments were sequenced. After sequencing, the nucleotide sequences of the transcribed constructs of the wild-type and mutant variants were established. Figure 1 shows the sequences of the obtained studied fragments of exon 9 of the *DOCK8* gene (capital letters) and adjacent exons of the plasmid (lower case letters) (the boundaries of the exons are separated by a red vertical line).

HEK293T cells (6×10^5 cells) were seeded in a 6-well plate in DMEM medium (NPP PanEco, Russia) with 10% FBS medium (Caprico Scientific, Germany) at 37 °C in an atmosphere of 5% CO2. Transfection of plasmids carrying mini-gene constructs, as well as "empty" plasmids, was performed in 6-well plates using the GenJect-39 reagent (Molecta, Russia) according to the manufacturer's protocol. 48 h after transfection, the cells were collected for extraction of total RNA using the Lira kit (Biolabmix, Russia). The amount of isolated total RNA was assessed using a Nano 500 spectrophotometer (Allsheng, China). Sample quality was assessed by measuring the following ratios: A260/280 ~1.8, A260/230 ~2.0-2.2. RNA integrity was assessed by using electrophoresis in 1% agarose gel. The obtained RNA samples were used to perform reverse transcription followed by PCR with primers flanking the mini-gene construct. Visualization of PCR products was performed using 1.5% agarose gel with ethidium bromide.

Results

According to WES data, no pathogenic variants were found in BC patients of the Buryat ethnic group. One of the 35 patients was found to have several rare variants of the causative genes, which we further studied and presented in this article.

A 52-year-old Buryat BC patient with a family history of BC (sister with BC) was found to have rare variants of unknown significance, *MLH1* c.C550T:p.R184C and FANCI c.A1111G:p.S371G with c.986C>T variant in a key immunity *DOCK8* gene (Table 1). The c.986C>T *DOCK8* variant was initially considered as secondary findings. In silico analysis indicated that *MLH1* c.C550T:p.R184C and FANCI c.A1111G:p.S371G variants do not affect splicing that limits the study of their clinical significance in vitro experiments.

The identified c.986C>T (rs75352090) variant of the *DOCK8* gene resides on chromosome 9 (chr 9:328113C>T (hg38)) and leads to an amino acid substitution at the (p.Ala329Val) protein level. According to the gnomAD database (v4.1.0), the minor allele frequency is 0.0003. However, in the ClinVar database, this substitution has been classified as a variant of conflicting significance. The c.986C>T (rs75352090) variant scored as 29.6 by CADD (Combined Annotation Dependent Depletion, a tool for

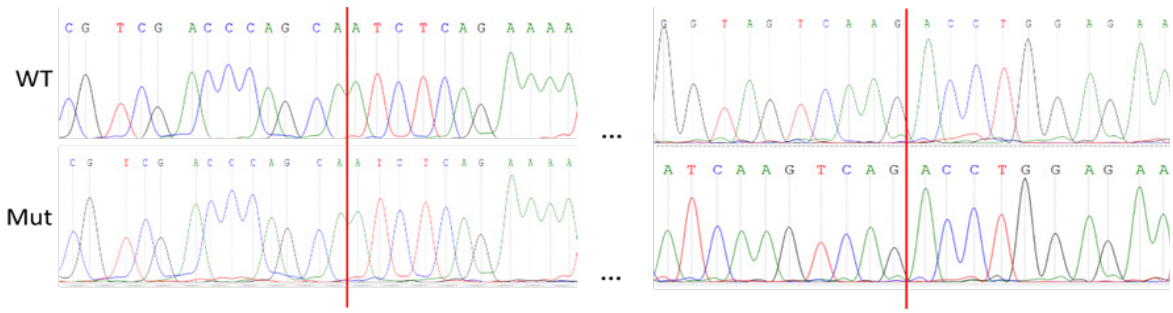

Figure 1. Sequences of the obtained studied fragments of exon 9 of the *DOCK8* gene: WT (cgacccagcaATCTCAGAAAATTTCACTGTGACCTGAACTCTGACCAGTTCAAAGGATTTCTGCGAGCTCACACGCCTTCAGTGGCCGCATCAAGTCAGGCGAGATCTGCAGTCTTCTCAGTCACCTACCCGTCCTCAGACATCTACCTGGTAGTCAAGacctggagaa) is the nucleotide sequence transcribed by the wild-type construct, Mut (cgacccagcaATCTCAGAAAATTTCACTGTGACCTGAACTCTGACCAGTTCAAAGGATTTCTGCGAGCTCACACGCCTTCAGTGGCCGCATCAAGTCAGacctggagaa) is the nucleotide sequence transcribed by the construct with the mutant variant (exon boundaries are separated by a red vertical line)

Table 1. Annotation of Variants Identified in a 52-Year-Old Buryat BC

Gene/ HGVS/ rs ID	CADD	REVEL	SpliceAI	Pangolin	PolyPhen	MAF, GnomAD	ClinVar
FANCM c.A4487G rs199514189	16.9	0.339	0	-0.02	0.355	0.00002	Uncertain significance
MLH1 c.C550T rs4986984	28.9	0.843	0.01	-0.04	0.996	0.00009	Conflicting classifications of pathogenicity Uncertain significance(2); Benign(6); Likely benign(3)
FANCI c.A1111G rs149008055	31	0.505	0	-0.01	0.689	0.0001	Conflicting classifications of pathogenicity Uncertain significance(1); Likely benign(1)
DOCK8 c.C782T rs75352090	29.6	0.196	0.96	0.78	0.887	0.0005	Conflicting classifications of pathogenicity Uncertain significance(2); Likely benign(3)

assessing the pathogenicity of genetic variants). Prediction of this genetic variant using an in silico tool revealed that it gives rise to a new donor splice site (SpliceAI DG: 0.960; Pangolin: 0.780) [12].

To assess the functional significance of c.986C>T variant of the *DOCK8* (p.Ala329Val), an approach using genetically engineered DNA constructs was chosen due to the unavailability of RNA extraction for transcriptome sequencing. DNA constructs represent a mini-gene, artificially-designed segment of DNA or vector that can be used to incorporate genetic material into a target tissue or cell.

We constructed the pSpl3-Flu2-TKdel vector carrying a *DOCK8* gene fragment flanked by introns 9 and 10 (Figure 2). After the construct assembly and its transformation into *E. coli*, we conducted screening of colonies containing a wild-type insertion and the potentially pathogenic variant (Figure 2A). The isolated plasmids were then transfected into the HEK293T cell culture. Two days later, total RNA was isolated, and reverse transcription was performed with DNase treatment. Next, PCR with primers specific to the flanking regions of the vector encoding fluorescent proteins (TurboFP365 and TagGFP2) was carried out.

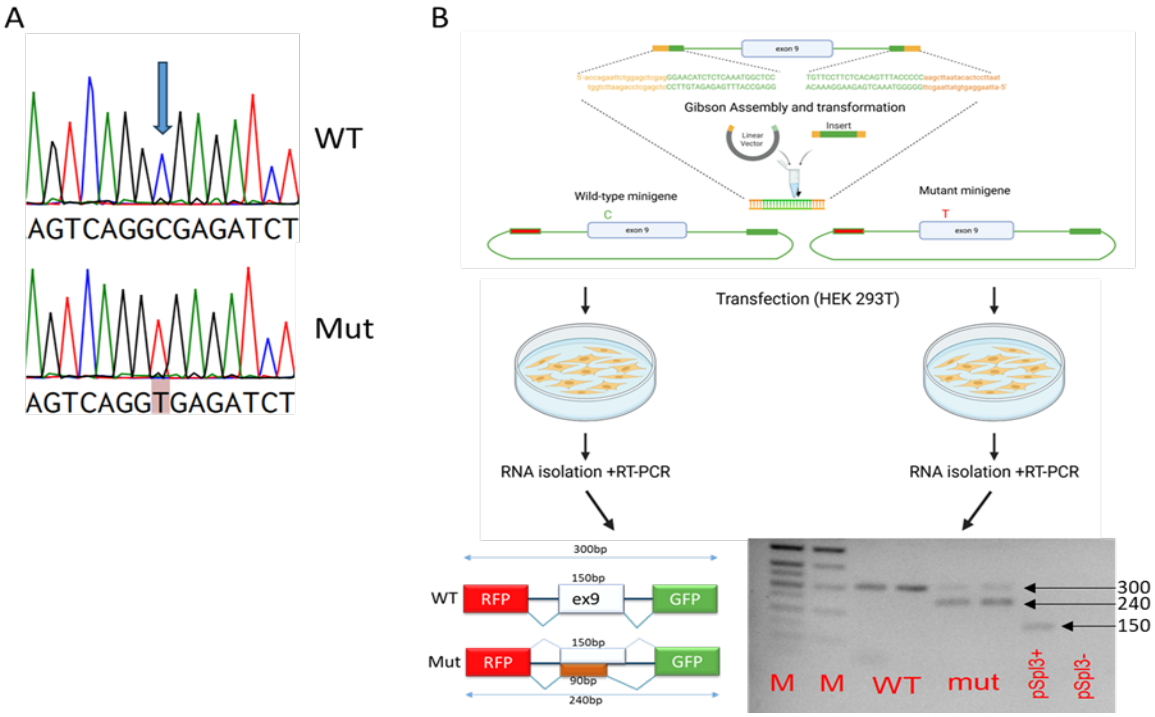

Figure 2. An analysis of the NM_203447.4:c.986C>T variant of the *DOCK8* gene using an in vitro DNA construct (mini-gene system). (A) plasmid sequencing data to detect the mutant and wild-type variants; (B) schematic representation of the pSpl3-Flu2-TKdel vector, the minigene assay protocol, and the results of electrophoretic separation of RT-PCR products in HEK293T cell lines: the cell line with a plasmid carrying the wild-type variant (WT); the cell line with a plasmid carrying the mutant variant (Mut); the cell line without plasmids (pSpl3-); the cell line with an empty plasmid (pSpl3+); the red and green rectangles correspond to the exonic sequences in the vector; the gray rectangle corresponds to exon 9 of the DOCK8 gene; the brown rectangle corresponds to the truncated fragment of exon 9.

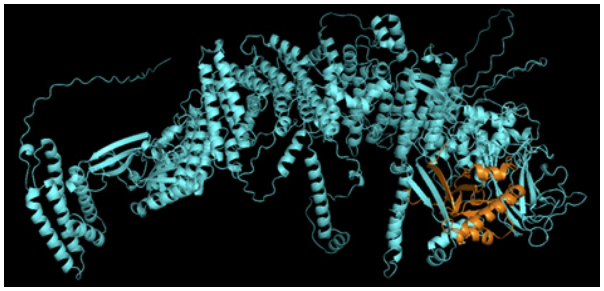

Figure 3. The Model of the DOCK8 Protein with a Deletion of 20 Amino Acids. The domain containing the deleted region is highlighted in orange.

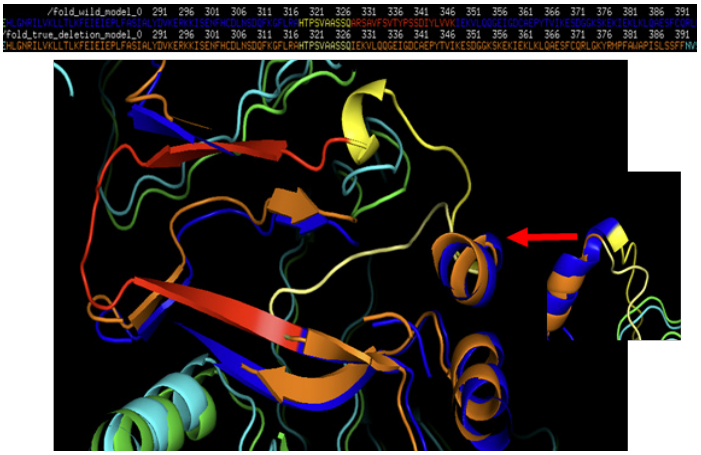

Figure 4. Structural Overlay of the Wild-Type and Deletion Mutant Fragments of *DOCK8.* The wild-type fragment (yellow) is shown on top. The region N-terminal to the deletion is shown in light yellow (bottom). A view from a different angle is shown on the right. The domain amino acid sequence is shown at the top.

Electrophoresis of PCR fragments obtained using cDNA as a template showed that in the presence of the c.986C>T variant, the length of the PCR product was 60 bp less than in the case of the reference sequence of this region.

In the presence of the c.986C>T, a non-canonical splice site variant in the DNA construct, two fragments are formed: a 300 bp-long product of exon 9 and a truncated fragment. The truncated PCR product was 60 bp shorter (with the total length of 240 bp), likely resulting in loss of a 20-amino acid fragment within the second domain of the *DOCK8* protein. The quantity of the truncated fragment being formed approximately twice that of the normal fragment (Figure 2B). Hence, the studied potentially pathogenic variant can affect exonic splicing enhancers, leading to aberrant RNA splicing.

Further, according to the AlphaFold2 tool, which allows modeling the spatial architecture of proteins, we have established that the deletion of 20 amino acids leads to a change in the structure of the protein [13]. This deletion is located near the conservative significant domain DHR-1 responsible for interaction with immunocompetent cells.

Thus, the presence of this mutation can lead to a violation of the function of an important conservative domain, father functional studies are required (Figures 3, 4).

Discussion

Currently, for indigenous groups such as the Buryats (Mongoloid ethnic group), the founder mutation in BC associated genes has not been definitively identified. The existing data are scarce, and treatment standards for HBOC in this ethnic group have not yet been established. In this regard, we initiated a study using WES to identify the full spectrum of genetic markers responsible for HBOC in Buryat patients.

In our study, a 52-year-old Buryat BC patient with a family history (sister has BC) was found to have rare variants of unknown significance (*MLH1* c.C550T:p.R184C and FANCI c.A1111G:p.S371G). *MLH1* (mutL homolog 1) is a tumor suppressor gene involved in DNA mismatch repair. It is well known that germinal mutations in this gene lead to Lynch syndrome (hereditary nonpolyposis colon cancer or ovarian cancer) [14]. FANCI (Fanconi anemia complementation group I) is a gene that encodes a protein that functions in DNA repair. Previous studies have highlighted the involvement of FANCI in various cancer types such as ovarian and BC [15]. In our study, the c.A1111G of the FANCI gene may be a BC causative variant with a low effect of penetration in a 52-year-old Buryat BC.

The c.986C>T *DOCK8* variant was initially considered as secondary findings, but later we studied it more closely. *DOCK8* (dedicator of cytokinesis 8) is a high-molecular-weight protein of approximately 190 kDa in size that plays a crucial role in the immune system, in particular in the function of T and B cells. *DOCK8* deficiency (MIM #243700) is a rare autosomal recessive disorder

leading to combined primary immunodeficiency [16]. Moreover, in accordance with literature, *DOCK8* could act as a tumor suppressor molecule and its deficiency is associated with the development of malignancies [17-20]. A study by Suzanne E. Aydin et al. (2015) examined 136 carriers of mutations in *DOCK8* and showed that early-onset malignancies were observed in 17% of cases [21]. Also, *DOCK8* deficiency is known to be associated with high mortality. According to Helen S. Su (2010), of the 32 patients with confirmed *DOCK8* mutations on both alleles, 7 died at a young age, and 3 died from malignancies (squamous cell carcinoma, T-cell leukemia-lymphoma) [17].

DOCK8 gene is located on chromosome 9 and consists of 48 exons. The majority of mutations affecting *DOCK8* are deletions that can range in size, most often resulting in absent protein expression (deletions, missense mutations). Specific mutant variants of *DOCK8* have been reported in certain populations, which may indicate a founder effect. It was shown that a mutation in the splice acceptor site IVS16: c.1869–1G>C as well as a large deletion spanning exons 1 to 27 were identified in Turkish patients. Mutation at position c.5625T>C: p.Y1875X was found in unrelated Saudi families [22]. In addition, a copy number alteration (CNA) of the *DOCK8* gene was identified in Tunisian patients with hereditary BC [23]. *DOCK8* deficiency is likely to be observed in consanguineous populations. The exact prevalence of *DOCK8* deficiency remains unknown; approximately 230–300 cases have been reported worldwide. Most patients with *DOCK8* deficiency are of Turkish or Arabic ethnicity; cases in North and South America, Europe, and China have also been reported [18].

In our study, the c.986C>T *DOCK8* variant was detected for the first time in BC patient of Buryat ethnicity with family history of cancer. The combined carriage of the c.A1111G mutation of the FANCI gene and c.986C>T *DOCK8* gene identified in this patient may likely increase the risk of developing BC. Our study has some limitations. A current assessment of the patient's immune status is not possible due to the fact that blood samples were collected more than ten years ago during pilot studies in the Republic of Buryatia.

However, for the first time in this study, using a DNA construct, it was established that the c.986C>T variant of the *DOCK8* gene (p.Ala329Val) results in the synthesis of truncated exon 9. Our functional data indicate a potential impact c.986C>T variant of the *DOCK8* gene on splicing. Our in vitro experiments identified a new clinically significant *DOCK8* c.986C>T variant, in addition to the 300 variants previously identified worldwide. However, the role of the c.986C>T variant in BC pathogenesis and its prevalence in Buryats ethnic group remain to be elucidated.

In conclusion, the combined carriage of the c.A1111G mutation of the *FANCI* gene and c.986C>T *DOCK8* gene identified in this patient likely increase the risk of developing BC. Our functional data indicate a potentional impact c.986C>T variant of the *DOCK8* gene on splicing. Our in vitro experiments identified a new clinically significant *DOCK8* c.986C>T variant, in addition to the 300 variants previously identified worldwide. The role of the c.986C>T variant in BC pathogenesis and its prevalence in Buryats ethnic group remain to be elucidated.

Author Contribution Statement

All authors contributed equally to the concept, literature search, writing manuscript, critical revision, and finalizing the manuscript.

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General

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Scientific Approval

The current study is the part of an approved student thesis

Ethical Declaration

The study was approved by the Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center (Protocol No. 10 of September 24, 2022). All patients signed informed consent to participate in this study.

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