IDENTIFICATION OF BRCA1/2 MUTATIONS IN BREAST CANCER PATIENTS BY NEXT-GENERATION SEQUENCING

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Breast cancer is one of the most widespread forms of solid tumors. By analyzing the traits of breast cancer pathogenesis at the molecular level using modern genetic analysis techniques and at different stages of the disease new data can be obtained to be further utilized in clinical practice. Molecular profiling based on next-generation sequencing is being increasingly applied as a clinical test to select target drugs for treating breast cancer patients with tumors highly resistant to therapy. In this study, we performed targeted sequencing of *BRCA1* and *BRCA2* oncogenes. In the total of 66 DNA samples from patients with breast tumors, BRCA1/2 mutations were found in 39 patients. There were 78 unique genetic variants, including 30 mutations in *BRCA1* and 48 mutations in *BRCA2*. We identified 33 mutations affecting the sites of post-translational modification in proteins (PMT mutations).

Keywords: *BRCA1*, *BRCA2*, breast cancer, NGS, DNA-sequencing, mutation, personalized medicine

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ИДЕНТИФИКАЦИЯ BRCA1/2-МУТАЦИЙ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ С ПРИМЕНЕНИЕМ ТЕХНОЛОГИИ ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ

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Рак молочной железы (РМЖ) является одной из наиболее распространенных форм солидных опухолей. Анализ особенностей патогенеза РМЖ на молекулярном уровне с применением современных методов генетического анализа и на разных стадиях заболевания позволяет получить новые данные для их дальнейшего применения в клинической практике. Молекулярное профилирование с применением технологий высокопроизводительного секвенирования все чаще применяют в качестве клинического теста при подборе таргетных препаратов для лечения пациентов с высокорезистентными к терапии опухолями при РМЖ. Целью работы было провести таргетное секвенирование генов *BRCA1* и *BRCA2* в составе панели онкогенов. Из 66 образцов ДНК пациентов с опухолями молочной железы, мутации BRCA1/2 обнаружены у 39 пацентов. Найдено 78 уникальных генетических вариантов, из них 30 мутаций в гене *BRCA1* и 48 мутаций в гене *BRCA2*. Идентифицировано 33 мутации, оказывающие влияние на сайты посттрансляционной модификации белков (PMT-мутации).

Ключевые слова: *BRCA1*, *BRCA2*, рак молочной железы, NGS, ДНК-секвенирование, мутация, персонализированная медицина

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Breast cancer (BC) is one of the most widespread forms of malignant neoplasms, next only to lung cancer and colorectal cancer. BC incidence has been growing in many parts of the world [1–4]. Early detection of the pathology and screening for BC is therefore a key task.

Suppressor genes *BRCA1* and *BRCA2* are important actors in regulating the signaling pathways associated with the functioning of DNA repair systems. Mutations in these genes entail an elevated risk of developing BC and some other forms of malignant tumors.

A substantial proportion of the mutations in tumors are somatic mutations, playing an important role both in the pathogenesis of sporadic BC and in the development of *de novo* resistance to anticancer drugs. Sporadic forms of cancer constitute, on average, 70–80% of BC cases, whereas only 10% of all the patients carry inherited mutations in the *BRCA1* and *BRCA2* genes [5].

The actual task of oncogenetics today is the development and improvement of approaches to the effective selection of anticancer drugs, taking into account the molecular-genetic features of tumor development.

The aim of this study was to identify the spectrum of mutations in the *BRCA1* and *BRCA2* genes in patients with BC by Illumina next-generation sequencing.

METHODS

Material for the study. Clinical characteristics of the patients

The collection of tumor samples for the study was taken from 66 patients with malignant breast neoplasms in hospital care at NN Blokhin National Medical Research Centre of Oncology of the Russian Health Ministry and Russian Scientific Center for X-ray Radiology of the Ministry of Health of the Russian Federation, (Moscow). The average age of the patients was 52.5 ± 9.7 years. The criteria for being included in the study were: age of 18 to 70, female, clinically verified BC diagnosis. Exclusion criteria: history of other forms of neoplasms, pregnancy. BC was staged according to TNM classification [6]. The study involved patients with stages T1–3N0–3M0–1. The study adhered to the principles of voluntariness and confidentiality. All patients provided informed consent to the study. The principal clinical characteristics of the patients are given in Table 1.

DNA isolation and quality control. Oncogene panel sequencing

Genomic DNA was isolated from tumor tissue samples by using DNeasy Blood and Tissue Kit (Qiagen; USA) as instructed by the manufacturer. The concentration of the extracted DNA specimens was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific; USA). The quality of the DNA samples was additionally tested by electorphoresis in 1% agarose gel with ethidium bromide.

DNA fragment libraries were prepared using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs; USA). The libraries were barcoded by PCR using two reagent kits: NEBNext Ultra DNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1, New England Biolabs; USA). DNA library quality control was done by measurements with Agilent Bioanalyzer 2100 (Agilent Technologies; USA) using High Sensitivity Kit as instructed by the manufacturer.

Coding regions of the tumor genome were enriched using MYbaits Onconome KL v1.5 Panel (Mycroarray; USA). The analysis was performed with a high-throughput genome sequencing system HiSeq 2500 (Illumina; USA) using paired 100-nucleotide reads. The samples were prepared and initiated according to Illumina protocols.

Bioinformatic processing of NGS data

Bioinformatic processing of the resultant NGS data was carried out using a previously developed algorithm [7, 8]. At first, the quality of the reads from DNA sequencing was assessed by Cutadapt software, and they were mapped to the reference genome hg19 (GRCh37.p13) by using the BWA tool (Burrows-

Table 1. Clinical characteristics of women with breast cancer (*n* = 66)

Parameter	Value, abs. no (%)
Age (years)	52.5 ± 9.7
Principal diagnosis:	
Left breast cancer	32 (48.5)
Right breast cancer	32 (48.5)
Bilateral cancer	2 (3)
Tumor T-stage (TNM classification):	
T1	36 (54.5)
T2	29 (43.9)
T3	1 (1.5)
Metastases in lymph nodes:	
without metastases, M0	56 (84.8)
with metastases, M1	10 (15.2)
Expression of estrogen receptors (ER):	
ER+	53 (80.3)
ER–	13(19.7)
Expression of progesterone receptors (PR):	
PR+	50 (75.8)
PR–	16 (24.2)
Expression of HER2/neu (Cerb-B2):	
Her2+	38 (57.6)
Her2–	28 (42.4)

Note: the values are in M ± SD or % form; T 1–3 — tumor stages according to TNM classification; ER — estrogen receptor expression; PR — progesterone receptor expression; HER2/neu (Cerb-B2) — expression of the human epidermal growth factor receptor *2*.

Wheeler Aligner). Paired reads were removed by running the specialized rmdup command in the SAMtools software package. Mutations in the NGS dataset were detected by MuTect, and DNA sequences covered by at least 12 readswere considered the most significant.

The mutation abundance was defined as the proportion (%) of mutation-supporing reads at a position. The functional effect of the mutations was assessed relying on ActiveDriverDB database [9]. The mutations affecting the coded protein were visualized using the ProteinPaint application [10].

RESULTS

We analyzed DNA samples from breast tumors (*n* = 66) for the presence of mutations in the *BRCA1* and *BRCA2* genes by Illumina next-generation sequencing. Bioinformatic processing of the NGS data revealed mutations in the *BRCA1* and *BRCA2* genes in 39 (59.1%) out of the 66 BC patients. Altogether 78 unique genetic variants were detected in the study, including 30 mutations in *BRCA1* and 48 mutations in *BRCA2*. Among all these mutations, 70 of the detected variants were identified as new mutations (89.7%). All the detected genetic variants are listed in the Table 2.

The highest frequency in the analysis was demonstrated by the mutations 17:41246746:T>C in *BRCA1* gene (52%) and 13:32914688:G>T in *BRCA2* gene (47%). The mutation 13:32910800:A>C in *BRCA2* gene occurred the most frequently among all samples, being identified in 10.7% (*n* = 3/28) of tumors with *BRCA2* mutations. Mutations in both *BRCA1* and *BRCA2* were found in 11 patients with BC (16.7%; *n* = 66).

Annotation against databases revealed 33 mutations (42.3%) influencing the sequence of the coded protein, including 16 in *BRCA1* gene and 17 in *BRCA2* gene. The

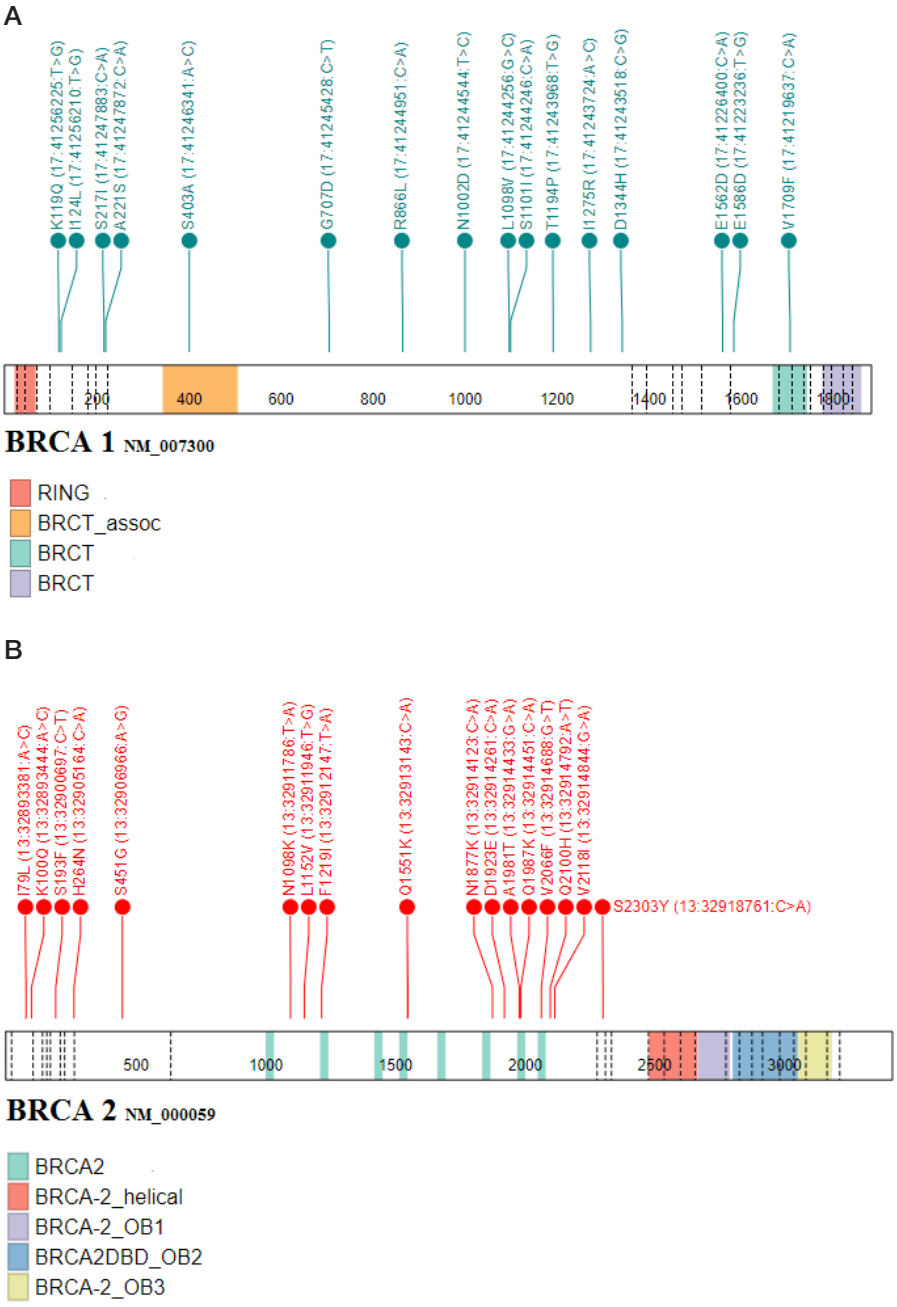

Fig. 1. The spectrum of mutations* affecting post-translational modification sites of proteins (PMT-mutations) in the genes *BRCA1* (**A**) and *BRCA2* (**B**), in patients with breast cancer (*n* = 39). * — based on mutation effect prediction according to ActiveDriverDB

Table 2. *BRCA1* and *BRCA2* mutations identified in patients with BC

Gene	Sample ID	BC Stage	The proportion of cancer cells in the sample[1], %	Variant allele frequency, %	Coverage at the point	PTM-mutation	Effect[2]	Reference number[2]	Canonical designation
BRCA1	1	IIIA	20	52	235	No	None	Novel	17:41246746:T>C
	2	IIA	9	30	117	No	None	rs1800744	17:41226488:C>A
	3	IA	70	4	106	No	None	Novel	17:41251858:T>G
				3	115	Yes	distal	Novel	17:41223236:T>G
				2	267	Yes	proximal	Novel	17:41243968:T>G
				1	439	No	None	Novel	17:41245560:C>A
	4	I	30	4	116	Yes	proximal	rs80357088 (dbSNP)	17:41247872:C>A
				1	685	Yes	proximal	rs80357192 (dbSNP)	17:41245428:C>T
	5	IIA	8	4	230	Yes	network-rewiring - motif loss	Novel	17:41244256:G>C
	6	I	90	2	169	Yes	direct	Novel	17:41244246:C>A
	7	IIA	21	2	250	No	None	Novel	17:41244207:T>C
	8	IA	8	2	306	No	None	Novel	17:41246576:A>C
	9	IA	6	2	270	Yes	distal	Novel	17:41243724:A>C
	10	IA	32	2	142	Yes	proximal	Novel	17:41256210:T>G
				2	142	Yes	distal	Novel	17:41256225:T>G
				1	333	Yes	direct	Novel	17:41246341:A>C
	11	IIB	95	2	166	Yes	proximal	BRCA (TCGA MC3)	17:41243518:C>G
				1	467	No	None	Novel	17:41245516:C>A
	12	IA	98	1	202	Yes	distal	Novel	17:41247883:C>A
	13	IA	15	1	660	Yes	network-rewiring - motif loss	Novel	17:41244951:C>A
	14	IIB	35	1	444	No	None	Novel	17:41245785:C>A
	15	I	12	1	569	No	None	Novel	17:41245228:C>T
	16	IIB	12	1	351	No	None	Novel	17:41245832:T>G
	17	IIA	57	1	413	No	None	Novel	17:41245859:C>A
	18	IA	12	1	211	Yes	proximal	Novel	17:41226400:C>A
	19	IIA	38	1	342	Yes	distal	rs786202665 (dbSNP)	17:41244544:T>C
	20	IIA	32	1	307	No	None	Novel	17:41246752:C>A
	21	IA	35	1	336	Yes	distal	Novel	17:41219637:C>A
				1	379	No	None	Novel	17:41246125:T>A
	22	IIA	10	1	390	No	None	Novel	17:41245026:C>A
BRCA2	9	IA	6	47	189	Yes	distal	Novel	13:32914688:G>T
				2	210	Yes	distal	Novel	13:32905164:C>A
	12	IA	98	6	471	No	None	rs28897716 (dbSNP)	13:32911295:G>A
				1	434	Yes	proximal	Novel	13:32893381:A>C
	23	IA	15	4	100	No	None	Novel	13:32906550:T>C
	24	I	50	3	63	No	None	rs55924966 (dbSNP)	13:32929408:G>A
	19	IIA	38	3	152	No	None	Novel	13:32912843:G>T
				2	276	No	None	Novel	13:32912258:C>A
	25	IA	65	2	319	No	None	Novel	13:32910800:A>C
	14	IIB	35	2	316	Yes	direct	rs864622305 (dbSNP)	13:32900697:C>T
	7	IIA	21	2	251	No	None	Novel	13:32944694:G>T
	26	IA	10	2	255	No	None	Novel	13:32911260:A>T
				1	537	No	None	Novel	13:32910800:A>C
	27	IIA	11	2	87	Yes	proximal	Novel	13:32918761:C>A
				2	238	No	None	Novel	13:32930703:C>A
	28	IIA	70	2	130	No	None	Novel	13:32931930:G>T
				1	307	Yes	distal	Novel	13:32914451:C>A
	29	I	1	2	262	No	None	Novel	13:32910800:A>C

End of Table 2

	30	IIA	55	2	200	No	None	Novel	13:32911499:C>A
				2	237	No	None	Novel	13:32913030:A>C
				1	274	Yes	distal	Novel	13:32914261:C>A
	10	IA	32	2	99	Yes	distal	Novel	13:32893444:A>C
				2	179	No	None	Novel	13:32907009:T>G
				2	188	Yes	proximal	Novel	13:32911946:T>G
	4	I	30	2	807	No	None	Novel	13:32914484:C>A
				1	323	No	None	Novel	13:32899216:G>A
				1	747	No	None	Novel	13:32915036:A>T
				1	279	No	None	Novel	13:32930596:T>A
				1	285	No	None	Novel	13:32930604:A>G
	21	IA	35	2	332	No	None	Novel	13:32914234:C>A
				1	440	No	None	Novel	13:32907309:C>A
	22	IIA	10	2	164	No	None	Novel	13:32912375:C>A
				1	298	No	None	Novel	13:32907051:A>T
				1	358	Yes	distal	Novel	13:32914844:G>A
	2	IIA	9	2	165	No	None	Novel	13:32968849:T>C
	31	IIIC	18	1	405	No	None	Novel	13:32913099:A>C
				1	266	No	None	Novel	13:32929173:C>A
	32	IIA	9	1	526	Yes	proximal	Novel	13:32913143:C>A
				1	399	No	None	Novel	13:32968988:C>A
	33	IIIA	10	1	233	Yes	distal	Novel	13:32911786:T>A
	14	IA	15	1	362	No	None	Novel	13:32936764:C>A
	34	IIA	14	1	371	Yes	distal	Novel	13:32912147:T>A
	35	IB	5	1	246	No	None	Novel	13:32913558:C>T
				1	434	Yes	distal	Novel	13:32914792:A>T
	36	IIB	25	1	344	Yes	distal	Novel	13:32914433:G>A
	37	IIB	80	1	569	Yes	distal	rs374326934 (dbSNP)	13:32914123:C>A
				1	275	No	None	Novel	13:32937605:G>A
	1	IIIA	20	1	320	Yes	distal	Novel	13:32906966:A>G
	38	I	10	1	439	No	None	Novel	13:32913444:C>A
	39	IIA	18	1	262	No	None	Novel	13:32930600:C>A
				1	363	No	None	Novel	13:32936793:C>A

Note: [1] — based on histological data; [2] — based on ActiveDriverDB data (https://www.activedriverdb.org/).

mutations affecting the sites of post-translational modification in proteins (PMT mutations) are shown in the Fig. 1.

DISCUSSION

Personalized targeted therapy is gaining ground in modern oncology. The development of a highly sensitive and cost-efficient approach to affordable routine diagnosis of tumors is therefore a priority task.

The “gold standard” for mutation detection today is Sanger sequencing, but its diagnostic capabilities are limited compared to next-generation genetic analysis systems. Tumor cells are histologically and genetically heterogeneous, contributing to the advantage of NGS-based techniques, which allow developing efficient bioinformatics pipelines for detecting genetic variants both in pairs of tumor and normal tissues samples and within individual biopsies containing a fraction of normal cell DNA.

Mutations in the key BC oncogenes *BRCA1* and *BRCA2* are among the most frequent and significant molecular aberrations, whose analysis can help in assessing the risk of tumor development, clinical prediction for BC patients, and in predicting the effectivenesss of anticancer drug therapy.

The *BRCA1* gene was identified by Y. Miki et al. in 1994 by positional cloning on the long arm of chromosome 17. The second gene — *BRCA2*, was mapped and isolated on chromosome 13q. *BRCA1* and *BRCA2* are suppressor genes, characterized by the autosomal dominant inheritance pattern and high penetrance. Recent molecular studies of *BRCA1* and *BRCA2* have demonstrated a wide spectrum of mutations present in these genes [5].

The international COSMIC database [11] contains over 900 somatic coding mutations of the *BRCA1* gene and over 1400 coding mutations of the *BRCA2* gene. A substantial part of these mutations result in structural transformations modifying

the function of protein products, thus undermining the capacity of repair systems to effectively fix DNA lesions. Many of the mutations in *BRCA1/BRCA2* are missence mutations, where the coding sequence is altered and one functional codon is changed to another.

Having analyzed the NGS data for the BC tumors in our study by bioinformatics techniques, we identified 78 unique mutations in the genes *BRCA1* and *BRCA2.* A majority of the mutations were found in *BRCA2*. According to the literature, the frequency of mutations differs notably between the genes *BRCA1* and *BRCA2* [5].

Further analysis using ActiveDriverDB showed that a large part of the genetic variants produce a functional effect on post-translational modification sites of the coded proteins (Fig. 1). Our study revealed 33 PMT-mutations, many of them previously unannotated. To confirm the pathogenic variants detected in the study and the status of the mutations, the research results need to be verified by Sanger sequencing using normal tissue samples.

CONCLUSIONS

Targeted next-generation sequencing appears to be the most promising approach for molecular profiling of tumors for clinical application. An integrated NGS-based analysis of mutations in the genes *BRCA1* and *BRCA2* in BC patients enables the identification of a greater number of mutations, including low mutant allele frequency variants, as well as genetic variants in biopsy samples with low tumor cell content. NGS-based approaches revealing mutations in the entire *BRCA1* and *BRCA2* coding sequence will enable a more effective identification of the patients to whom an adequate therapy with targeted anticancer drugs can be administered.

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