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CLINICAL SIGNIFICANCE OF *BRCA1* GENE SEQUENCING AND ITS PROMOTER METHYLATION TESTING IN THE SEARCH STRATEGY FOR THERAPEUTIC TARGETS IN BREAST CANCER TREATMENT

Background. Currently, there is a great interest in the genetic testing of *BRCA1* and *BRCA2* due to the fact that for patients with breast cancer (BC) with pathogenic variants of these genes, the use of the PARP inhibitors could be also provided in addition to implemented treatment protocols. **The aim** of this study was to characterize the molecular genetic structure of the *BRCA1* gene in BC patients without progenitor germline mutations taking into account the methylation state of the promoter region. **Materials and Methods**. The study involved 210 patients with newly diagnosed BC. The most common germline pathogenic variants of the *BRCA1* (185delAG, 5382insC, 4153delA, T300G) and *BRCA2* (6174delT) genes were identified in the peripheral blood. A subgroup of 14 patients without progenitor pathological variants of the *BRCA1* and *BRCA2* genes and with a family history of cancer was randomly selected. For them, *BRCA1* gene sequencing by Sanger and hypermethylation of the *BRCA1* gene promoter region were analyzed. **Results**. The following frequencies of *BRCA1* mutations were determined in the general group: 5382insC – 8.6%, 4153delA – 0.5%, T300G – 0.5%. The analysis of the *BRCA1* gene by Sanger sequencing revealed 11 *BRCA1* gene variants in 10 out of 14 BC patients. All of them, according to the currently available data, were defined as "benign" and not clinically relevant. The frequency of the detection of hypermethylation of the BRCA1 gene promoter region in the randomly selected group of patients was 14.3%. **Conclusions**. In BC patients, not only common mutations but

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also the methylation status of the *BRCA1* gene promoter region in the peripheral blood should be determined. The whole-genome sequencing of the *BRCA1* gene may be the last step in determining the genetic characteristics of BC patients carried out to optimize the treatment and improve survival thanks to the higher prevalence of the progenitor mutations and hypermethylation of the *BRCA1* gene promoter.

Keywords: breast cancer, *BRCA1*, *BRCA2*, 5382insC, sequencing, hypermethylation.

BRCA1 and *BRCA2* are known tumor suppressor genes. The pathogenic variants in these genes lead to impaired repair processes and an increased risk of developing cancer throughout life. These pathological variants are characterized by a high penetrance. In particular, the risk of developing breast cancer (BC) before the age of 70 is 64.6% for the carriers of the mutations in the *BRCA1* gene and 61.0% for the carriers of the mutations in the *BRCA2* gene [1]. As a rule, the pathogenic variants of the *BRCA1* and *BRCA2* genes are detected in the heterozygous state in the peripheral blood, and cases with a combination of several pathogenic variants in one patient are rare. There are reports on familial cases of BC in Italy, in which a combination of the pathogenic variants was detected during a blood test in patients with a significant family history of cancer [2 3].

The pathogenic variants of the *BRCA1* and *BRCA2* genes are divided into germline (inherited and associated with an increase in the risk of cancer) and somatic (which can occur in the target organs during the development of cancer). Most often, the pathogenic variants of the *BRCA1* and *BRCA2* genes were detected in triple-negative BC patients, so for a long time, the detection of such variants was recommended only in cases of a family history of cancer and in triple-negative BC. However, over time, the number of criteria for genetic testing of the *BRCA1* and *BRCA2* gene variants in BC patients has been expanded [4]. Therefore, testing for these variants has gone beyond the protocols for patients with triple-negative BC. Most protocols, as a rule, identify pathogenic variants of the *BRCA1* and *BRCA2* genes in the blood and in the target organs determining whether this variant is somatic or germline [4]. The great interest in genetic testing of *BRCA1* and *BRCA2* is caused by the fact that for patients with pathogenic variants of these genes, in addition to the implemented treatment protocols, the use of PARP inhibitors is also provided [5].

In this regard, the study of the structure of the *BRCA1* gene and its functional activity is of particular interest because the large rearrangements of the gene [6] could disrupt its functional activity in the same way, or even more, as the pathogenic variants. In particular, the hypermethylation of the *BRCA1* gene promoter region may be more widespread compared to the frequency of most common pathogenic variants of the *BRCA1* gene as such [7]. As shown in previous studies, hypermethylation is not "tied" to the triple-negative BC and is not detected more often in patients with a family history of cancer, but at the same time can be inherited [7, 8]. However, the clinical attention to making a diagnosis and choosing the optimal treatment protocol is focused on the detection of germline mutations and the search for the pathological variants of *BRCA1* detected by gene sequencing [4]. Less attention has been paid to analyzing the prevalence of non-pathological, especially other, including benign, variants of the *BRCA1* gene in BC patients without progenitor germline mutations.

Therefore, the aim of this study was to characterize the molecular genetic structure of the *BRCA1* gene in BC patients without progenitor germline mutations taking into account the methylation state of the promoter region.

Materials and Methods

The study involved 210 patients (mean age 47.8 ± ± 13.2 years) with newly diagnosed BC who were treated at the Department of Oncology of

the Bohomolets National Medical University at the Kyiv City Clinical Oncology Centre. In accordance with clinical standards, all patients underwent the histological examination of tumor tissue and immunohistochemical analysis of tumor samples. When taking anamnesis, special attention was paid to the factors indicating a possible hereditary nature of the disease, and a family tree was drawn up. The peripheral blood samples were taken for the molecular genetic analysis.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Commission on Bioethical Expertise and Research Ethics of Bogomolets National Medical University (0120U100871). All patients provided their informed consent to participate in the study.

Using the standard molecular genetic methods (polymerase chain reaction and polymerase chain reaction with restriction analysis), the most common germline pathogenic variants of *BRCA1* (185delAG, 5382insC, 4153delA, T300G) and *BRCA2* (6174delT) genes were identified in the peripheral blood of patients [7].

A subgroup of 14 patients without progenitor pathological variants of the *BRCA1* and *BRCA2* genes and with a family history of cancer (2—5 malignant neoplasms of the female reproductive system in first- to third-degree relatives) was randomly selected.

For the randomly selected subgroup of patients with no progenitor pathogenic variants and family history, *BRCA1* gene sequencing was performed in the peripheral blood samples by Sanger using a 3130 Genetic Analyzer (Applied Biosystems, USA) at the Department of Molecular Oncogenetics of the Institute of Molecular Biology and Genetics, NAS of Ukraine. The Sanger sequencing data were reviewed using the software Chromas v.2.6.6. The genomic alterations were identified by alignment to the reference genome GRCh38 (hg38). In addition, the hypermethylation of the *BRCA1* gene promoter region was determined in patients of this subgroup, for which the bisulfide conversion of DNA isolated from peripheral blood and methyl-specific polymerase chain reaction were performed [9].

Results

In the total group of patients diagnosed with breast cancer (n = 210), the pathogenic *BRCA1* variants included in the "standard" diagnostic panels were detected in 20 (9.5%) women (Table 1). It was found that the most common pathogenic variant of the *BRCA1* gene was 5382insC. Its prevalence was 8.6%, i.e. it was detected in approximately 1 of 12 BC patients. The frequency of detecting two other pathogenic variants T300G and 4153delA was extremely low.

The analysis of the *BRCA1* gene by Sanger sequencing revealed 11 *BRCA1* gene variants in 10 out of 14 BC patients. The characteristics of the identified *BRCA1* gene variants and their frequency of occurrence are shown in Table 2.

As seen in Table 2 the identified variants, according to the data available to date, are defined as "benign" and not clinically relevant.

Table 3 shows the clinical and molecular genetic characteristics of the randomly selected BC patients.

Table 1. **Prevalence of the most common *BRCA1* and *BRCA2* gene mutations in the group of BC patients**

Gene	Pathogenic variant	Number of patients with pathogenic variant	Frequency of pathogenic variant
BRCA1	185delAG	0	0
BRCA1	5382insC	18	8.6%
BRCA1	4153delA	1	0.5%
BRCA1	T300G	1	0.5%
BRCA2	6174delT	0	0
Total		20	9.5%

As shown in Table 3 no *BRCA1* gene variants were detected in 4 patients; in one patient, a heterozygous genotype for the c.1067A>G variant was determined; the remaining 9 patients had combinations of genotypes for various non-pathogenic variants. 6 out of 14 (42.3%) patients had triple-negative BC, and the combinations of the benign *BRCA1* gene variants detected in 4 of them covered exons 11, 13, and 17 and in 2 — non-coding introns 7 and 18. One of the patients with triple-negative BC had hypermethylation of the *BRCA1* promoter in addition to the combination of the benign gene variants. Among the other 8 out of 14 (57.1%) patients with estrogen receptor-positive BC, two patients had no mutations and no *BRCA1* promoter hypermethylation. The combination of the benign gene variants, like in the case of triple-negative BC, was detected in exons 11, 13, 17, and in the non-coding intron 18 of the *BRCA1* gene, and hypermethylation of the gene promoter was also detected in one of the patients. Both cases of hypermethylation of the *BRCA1* promoter were detected in patients with a combination of benign gene variants in exons 11, 13, 17 and in the non-coding intron 18 of the *BRCA1* gene. In general, the frequency of detection of hypermethylation of the *BRCA1* gene promoter region in the randomly selected group of patients was 14.3%.

Discussion

BC is one of the most common cancers in women, and its incidence is steadily increasing worldwide. Over the past few decades, new

Table 2. **Characteristics and frequency of the identified *BRCA1* gene variants**

No	Exon/ Intron	*HGVS cDNA*	*dbSNP*	*Amino acid change*	*Mutation type*	Clinical importance		Number of patients (genotype), n, %
						BIC	ClinVar	
1	In7	c.442-34C>T	rs799923	Non-coding	Intronic	No	Benign	1 (CT) 7.1
2	Ex11	c.1067A>G	rs1799950	p.Gln356Arg	Missense	Unknown	Benign	2 (AG) 14.3
3	Ex11	c.2077G>A	rs4986850	p.Asp693Asn	Missense	No	Benign	3 (GA) 21.4
4	Ex11	c.2082C>T	rs1799949	p.Ser694Ser	Synonymous	No	Benign	4 (CT) 28.6 5 (TT) 35.7
5	Ex11	c.2311T>C	rs16940	p.Leu771Leu	Synonymous	No	Benign	4 (TC) 28.6 4 (CC) 28.6
6	Ex11	c.2612C>T	rs799917	p.Pro871Leu	Missense	No	Benign	2 (TC) 14.3 4 (TT) 28.6
7	Ex11	c.3113A>G	rs16941	p.Glu1038Gly	Missense	No	Benign	4 (AG) 28.6 5 (GG) 35.7
8	Ex11	c.3548A>G	rs16942	p.Lys1183Arg	Missense	No	Benign	4 (AG) 28.6 5 (GG) 35.7
9	Ex13	c.4308T>C	rs1060915	p.Ser1436Ser	Synonymous	No	Benign	3 (TC) 21.4 5 (CC) 35.7
10	Ex17	c.4837A>G	rs1799966	p.Ser1613Gly	Missense	No	Benign	4 (AG) 28.6 5 (GG) 35.7
11	In18	c.5152+66G>A	rs3092994	Non-coding	Intronic	No	Benign	5 (GA) 35.7 4 (AA) 28.6

Table 3. **Clinical and molecular genetic characteristics of BC patients**

		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14
Age, years		35	32	34	42	57	56	49	67	55	52	80	52	66	51
TNM		T1N0M0	T2N0M0	T3N2M1	T2N0M0	T1N0M0	T1N0M0	T2N0M0	T1N0M0	T2N0M0	T2N0M0	T1N0M0	T2N0M0	T1N0M0	T1N0M0
ER		+	–	+	–	+	–	+	+	+	–	–	+	+	–
PR		+	–	+	–	+	–	–	+	+	–	–	+	+	–
HER-2/neu		–	–	–	–	–	–	–	–	–	–	–	–	–	–
BRCA1	c.442-34C>T														CT
	c.1067A>G	AG													AG
	c.2077G>A			GA			GA	GA							
	c.2082C>T			TT	TT		TT	TT	CT	CT	CT		TT		CT
	c.2311T>C			CC	CC		CC		TC	TC	TC		CC		TC
	c.2612C>T			TT	TT		TT		TC	TC	TC		TT		
	c.3113A>G			GG	GG		GG	GG	AG	AG	AG		GG		AG
	c.3548A>G			GG	GG		GG	GG	AG	AG	AG		GG		AG
	c.4308T>C			CC	CC		CC	CC	TC	TC			CC		TC
	c.4837A>G			GG	GG		GG	GG	AG	AG	AG		GG		GA
	c.5152+66G>A			AA	AA		AA	AA	GA	GA	GA		GA		GA
Methylation status of *BRCA1* promoter region		UU	UU	UU	UU	UU	UU	UU	UU	MU	MU	UU	UU	UU	UU

MU, hypermethylated; UU, unmethylated

approaches have been proposed to identify risk groups for the purpose of timely diagnosis and the selection of effective treatment regimens. The description of the hereditary tumor syndromes has made it possible to distinguish between sporadic, familial, and hereditary forms of BC, which differ significantly. In particular, the hereditary BC is characterized by young age of onset, a high risk of developing reproductive tumors in first-degree relatives, a high risk of developing tumors after hormonal contraception, *in vitro* fertilization and hormone replacement therapy, a very high incidence of triple-negative BC (ER-, PR-, HER-2/neu-), which is a marker of the unfavorable disease outcome, the differences in the extent of the surgery, the effectiveness of different treatment regimens, etc.

The discovery of the *BRCA1* and *BRCA2* genes in 1994–1995 allowed us to confirm the association of mutations in these genes with the occurrence of about 30% of familial cancer cases. As already mentioned, the *BRCA1* and *BRCA2* genes are highly penetrant suppressor genes with autosomal dominant inheritance within the same family. The *BRCA1* gene (OMIM*113705) is located on chromosome 17q12-21, has 24 exons, and is 110 kb in length. The BRCA1 protein is involved in numerous cellular processes such as cell cycle control, transcription, DNA damage repair, protein ubiquitination, etc.

Numerous population-based studies have identified a "founder effect" for some mutations. For example, the 185delAG and 5382insC mutations in the *BRCA1* gene and the 6174delT mutation in the *BRCA2* gene are found with an increased frequency among Ashkenazi Jews. In this population group, those three mutations account for more than 90% of all mutations detected in the *BRCA1/2* genes [10]. In Italy, in the region of Tuscany, more than 73% of all detected mutations in the *BRCA1* gene are the 3347delAG, 3404delA, 1499insA, and 5181delGTT mutations [11]. More than 68% of all detected mutations in one of the regions of Norway are 1675delA, 3347delAG, 816delGT, and 1135insA mutations in the *BRCA1* gene [12]. In this study and in previous studies, we have found that the most common mutation among BC patients in Ukraine is 5382insC in the *BRCA1* gene, which is a progenitor, and other progenitor pathogenic variants are rarely detected. In this study, T300G and 4153delA were detected in BC patients with the same frequency of 0.5%. According to the Ukrainian researchers, the progenitor mutations in BC patients in Ukraine occur with a frequency of 3.8% to 13%, which was noted in our previous work [7, 13].

As for the *BRCA1* gene sequencing results, we identified only benign (at the time of the study) variants. No pathogenic and/or clinically significant variants were identified. However, it should be noted that in 10 out of 14 patients, the above-mentioned benign variants of the *BRCA1* gene were identified in three exons and two non-coding introns, like in the case of triple-negative and estrogen-progesterone- positive cancers. This raises a question of how the combination of several benign variants can affect the functional activity of the gene. It is known that exon 11 of *BRCA1* is a large central exon and it represents 60% of the coding sequence [14]. This exon encodes protein signals that are necessary for nuclear localization of the gene product and therefore is considered relevant to cell cycle control and DNA damage repair [15]. Exon 11 is alternatively spliced in most cell and tissue types, including normal and BC cells, and across cell-cycle phases [15]. Ruiz de Garibay et al. [15] after a thorough study of the genetic and molecular factors that affect exon 11 splicing recognized that the unidentified variants and elements of the *BRCA1* gene might cooperate to determine exon 11 inclusion/exclusion and/or isoforms. Thus, the benign gene variants that we identified, due to their combination and exon damage, may be of important functional significance.

The hypermethylation of the *BRCA1* gene promoter region in BC was first reported in 1997 [16].

Later, it was shown that the hypermethylation of CpG sites near the *BRCA1* transcription start site was associated with a decrease in mRNA and protein [17]. As for the frequency of the hypermethylation of the *BRCA1* gene promoter among women with BC, it differs in various parts of the world. The frequency of the hypermethylation of the *BRCA1* gene promoter region determined in our study (14.3%) is close to the frequency among BC patients in Europe (18.8%), and does not differ significantly from the frequency of the hypermethylation in the peripheral blood of Ukrainian patients described in a previous study [7].

The clinical protocols of genetic diagnosing recommended for BC patients have not yet regulated the detection of hypermethylation and its use as a target, although the hypermethylation of the *BRCA1* promoter, as well as mutations of this gene, equally affects the intracellular repair processes [18, 19]. In addition, it is clear that the same targeted therapy can be used for promoter hypermethylation as for pathogenic variants of the *BRCA1* gene [20].

Basing on our and other studies [21], we consider that it is necessary to discuss the issue of building a genetic diagnostic algorithm in BC patients depending on the preliminary identification of progenitor pathogenic variants and hypermethylation of the *BRCA1* promoter, followed by gene sequencing and the identification and analysis of the functional significance of other (non-pathogenic) gene variants.

Therefore, in this study, the molecular genetic features of the pathogenic and benign gene variants have been determined for Ukrainian BC patients. The frequency of the detection of the progenitor pathogenic variants was 9.5% in the BC patients under study, and no other pathogenic variants of the *BRCA1* gene were detected upon the random selection. In the random sampling, the hypermethylation of the *BRCA1* promoter was detected with a frequency of 14.3%. The results indicate that in BC patients, not only the common mutations should be determined in the peripheral blood but also the methylation status of the *BRCA1* gene promoter region. The whole-genome sequencing of the *BRCA1* gene may be the last step in determining the genetic characteristics of BC patients to optimize the treatment and improve survival.

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КЛІНІЧНЕ ЗНАЧЕННЯ СЕКВЕНУВАННЯ ГЕНА *BRCA1* ТА ТЕСТУВАННЯ МЕТИЛЮВАННЯ ЙОГО ПРОМОТОРА В СТРАТЕГІЇ ПОШУКУ ТЕРАПЕВТИЧНИХ ЦІЛЕЙ У ЛІКУВАННІ РАКУ МОЛОЧНОЇ ЗАЛОЗИ

На сьогоднішній день існує великий інтерес до генетичного тестування *BRCA1* та *BRCA*, викликаний тим, що для пацієнток із раком молочної залози (РМЗ) з патогенними варіантами цих генів, окрім впроваджених протоколів лікування, передбачається і застосування PARp-інгібіторів. Тому **метою** даної роботи стала молекулярно-генетична характеристика структури гена *BRCA1* у пацієнток з РМЗ та відсутніми прогеніторними гермінативними мутаціями з урахуванням стану метилування промоторної ділянки. **Матеріали**

та методи: В дослідженні приймало участь 210 пацієнток з уперше діагностованим РМЗ. У периферійній крові пацієнток визначали найбільш поширені гермінативні патогенні варіанти генів *BRCA1* (185delAG, 5382insC, 4153delA, T300G) та *BRCA2* (6174delT). Серед пацієнток, у яких не було виявлено прогеніторних патологічних варіантів генів *BRCA1* та *BRCA2* та які мали онкологічно обтяжений сімейний анамнез, було рандомно обрано підгрупу із 14 пацієнток. Для них проведено секвенування гена *BRCA1* у зразках периферійної крові за Сенгером та визначення гіперметилування промоторної ділянки гена *BRCA1*. **Результати:** В загальній групі пацієнток визначено наступні мутації гена *BRCA1*: 5382insC – 8.6%, 4153delA – 0.5%, T300G – 0.5%. Аналіз гена *BRCA1* методом сиквенсу за Сенгером дозволив виявити 11 варіантів гена *BRCA1* у 10 з 14 пацієнток з РМЗ — усі вони, згідно з наявними на сьогодні даними, визначені як "доброякісні" та такі, що не мають клінічної значущості. Частота виявлення гіперметилювання промоторної ділянки гена *BRCA1* у рандомно обраній групі пацієнток становила 14,3%. **Висновки:** Отримані результати вказують на те, що в пацієнток з РМЗ в периферичній крові слід проводити не тільки визначення поширених мутацій, але й статус метилювання промоторної ділянки гена *BRCA1*. Повногеномне секвенування гена *BRCA1* може стати останнім етапом у визначенні генетичної характеристики пацієнтів з РМЗ, яка здійснюється для оптимізації лікування та покращення виживання, у зв'язку з більшою поширеністю прогеніторних мутацій та гіперметилування промотору *BRCA1*.

Ключові слова: рак молочної залози, *BRCA1*, *BRCA2*, 5382insC, секвенування, гіперметилювання