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Epigenetic markers-based breast cancer early detection method development

Relevance: According to the International Agency for Research on Cancer (IARC), breast cancer ranks 1st-2nd among other cancers globally [1], including Kazakhstan [2]. In Kazakhstan, the annual growth in breast cancer incidence exceeds 26.6%. In 2018-2019, breast cancer was the 3rd most common cause of cancer death in Kazakhstan, accounting for 8.7-8.1 percent, respectively. Early detection of breast cancer remains an acute issue. In particular, early detection should be improved. Epigenetic studies of cancer patients confirm that epigenetic biomarkers could be used as early cancer diagnostic markers, including breast cancer.

The study aimed to find specific diagnostic markers by methylation profiling of peripheral blood mononuclear cell (PBMC) DNA in breast cancer patients.

Results: Plasma samples of the Kazakhstani population with breast cancer possessed mononuclear cell methylation markers in CpG islets associated with JAM3, C17orf64, MSC, and C7orf51 genes and the CpG islet associated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434, which were missing in healthy individuals. These biomarkers allow differentiating breast cancer from other cancers with a specificity of 0.91 and a sensitivity of 0.94 compared to methylation data from open DNA methylation databases (for Illumina 450K): TCGA, GSE40279, GSE61496, GSE76269 u GSE66836.

Conclusion: Early breast cancer detection method using peripheral blood mononuclear cell DNA methylation profile, namely in CpG islets associated with JAM3, C17orf64, MSC, and C7orf51 genes and the CpG islet associated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434 is enough specific and sensitive to use it in breast cancer screening.

Keywords: breast cancer (BC), epigenetics, DNA methylation, peripheral blood mononuclear cells (PBMC), early detection, biomarker.

Introduction: According to the International Agency for Research on Cancer (IARC), breast cancer (BC) ranks 1st-2nd among other cancers globally [1]. More than 4000 BC cases are detected in the Republic of Kazakhstan each year. Thus, 4955 new BC cases were reported in 2019. In the same year, BC ranked 3rd in cancer mortality, amounting to 8.1% of all cancer deaths [2]. Despite significant BC awareness and screening examinations in Kazakhstan, early detection remains quite low. Therefore, the issue of early diagnostics of primary BC before relapses and metastases shall be solved to increase the therapy efficacy.

The advances in molecular biology give a real opportunity to diagnose the onset of the tumor process by simultaneously assessing the expression of thousands of genes that control cellular growth, differentiation, and apoptosis. Thus, the gene expression analysis has revealed five BC subtypes, such as luminal A, luminal B, HER2-enriched, basal-like, and claudin-low. Each of them has unique biological and prognostic features [3]. Another research analyzed 13 BC biomarkers' categories, including six new proposed by the authors [4]. Some indicators were recommended for practical

use: CA 15-3, CA 27.29, carcinoembryonic antigen, estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, urokinase plasminogen activator, plasminogen activator 1 inhibitor, and certain multivariable gene expression assays [3, 4]. However, it is known that BC, as well as other cancers, development is promoted by progressive accumulation of not only genetic but also epigenetic changes, including point mutations in individual genes, inactivation of tumor suppressor genes, amplification, recombination of chromosome regions, changes in their methylation (to abnormal) [5, 6].

Certain transformations in gene expression are involved in many malignancy-related processes [7, 8]. Some of these transformations are due only to dynamic epigenetic modifications manifested in a change in the cytosine-guanine DNA islands (CpG dinucleotides) methylation status [9-11]. At that, epigenetic DNA alterations in peripheral blood mononuclear cells (PBMC) are often linked to immune disorders related to malignancy development [12, 13].

A review of epigenetic research for cancer diagnostic purposes using BC patients' cancerous tissue or

peripheral blood has revealed several promising epigenetic biomarkers for possible early BC detection [6]. Different authors in different countries studied the DNA methylation status of CpG islets (inside and outside the islands) of various genes, including those associated with the risk of developing BC [7-10]. Brennan et al. suggested using DKK3 and ITIH5 methylation in patients' blood during periodic screening as BC biomarker (in case of breast glandular tissue increased density). They found significant hypermethylation in CpG islets' DKK3 and ITIH5 in women with BC compared to healthy women and patients with benign tumors [11].

A study of miRNA genes' CpG islets' methylation in 62 BC patients with epithelial tumors revealed increased methylation frequency in 5 genes: miRNA-9-1, miRNA-9-3, miRNA-34b / c, miRNA-193a, miRNA-129-2. This result correlated with some clinical and molecular tumor features and differed significantly from the same parameters in the adjacent histologically unchanged breast tissue [14]. Yang et al. found significant methylation in hyaluronoglucosaminidase 2 (HYAL2) CpG islets in BC patients' peripheral blood (with a sensitivity of 64% and a specificity of 90% versus healthy participants) not associated with circulating tumor DNA. This could evidence a possible use of methylation in early BC diagnostics when examining not only the tumor but also the peripheral blood of patients [9]. In a study of hypermethylation status of a kinesin 1A family (KIF1A) member promoter in the plasma of BC patients, Guerrero-Preston et al. also showed the ability to distinguish blood plasma samples from Patients with BC from samples with no cancer [10].

The prerequisite for this method development was the need to determine reliable diagnostic BC markers before visual detection of the disease. However, specific features of peripheral blood cells in BC patients that would distinguish them from healthy cells have not been investigated yet. Our hypothesis based on preliminary research suggests specific peripheral blood cells' DNA methylation profile in BC that could serve as the earliest BC biomarkers.

The study aimed to find specific diagnostic markers by methylation profiling of peripheral blood mononuclear cell (PBMC) DNA in breast cancer patients.

Materials and Methods: The search for BC markers based on PBMC methylation profile included 101 patients examined and treated for BC at the Kazakh Institute of Oncology and Radiology (Almaty, Kazakhstan). The examination followed the Protocol of the scientific

and technical program No. AP05131940 «Possibilities of determining T-lymphocytes' markers in early diagnosis and prognosis of lung cancer and BC» within the budget program 217 «Science Development,» sub-program 102 «Research Grant Funding.» All patients had histologically verified diagnoses.

The inclusion criteria: patients of pre- and postmenopausal age with early, locally advanced, and disseminated forms of BC administered to receive specialized antitumor treatment as per international treatment protocols.

The exclusion criteria: any identified inflammatory disease (bacterial or viral infection), diabetes, asthma, autoimmune disease, or acute thyroid disease that may alter immune performance.

The control group consisted of 50 healthy women. The analysis also utilized methylation data of open DNA methylation databases (Illumina 450K): TCGA (cancer genome atlas), GSE40279, GSE61496, GSE76269, and GSE66836.

15.0 ml of the patient's blood were collected into VenoSafe EDTA tubes. Lymphocytes and plasma were isolated from whole blood using ficoll. Before the molecular genetic analysis, the samples were cryopreserved in a freezer at -20°C. Circulating DNA was isolated from the top plasma cell-free layer, genome DNA – from the lymphocyte layer. Plasma DNA was extracted using DNA extraction “cell-free protocol” (HKG Epitherapeutics, Hong-Kong), followed by conversion to bisulfite using an EZ DNA Methylation-Direct kit (Zymo Research, CA, USA) and cryopreservation (at -20 °C) before further processing.

Samples were sequenced using an Illumina MiSeq sequencer (Illumina, CA, USA) by DNA methylation using Infinium Methylation Epic bead array. Two PCR cycles were performed for multiplex sequencing. The first cycle aimed to study CpG 5 islets of the AM3, C17orf64, MSC, and C7orf51 genes and CpG islet associated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434, which were methylated in all studied BC cells. In the first stage of amplification, the primers' sequence corresponded to the bisulfite-converted sequence, which served as an anchor for the second PCR. At the second stage, we added a primer that determined the index sequence in sequencing individually for each patient. The first PCR amplification cycle was carried out in reaction mixtures containing 2 µl of bisulfite-converted DNA and Taq DNA polymerase (Thermo Scientific, CA, USA), us-

ing a set of reverse and forward primers for selected genes using standard amplification. The second PCR cycle was carried out to mark each amplicon with a specific barcode using a series of forward and reverse HKG barcode sets. The second PCR results were used for quality control.

The library containing the same dose of the obtained amplicons was collected based on their dsDNA concentrations determined with the Qubit™ HS dsDNA assay kit (Invitrogen, CA, USA), then cleaned twice using cleaning balls, and quantified by RealTime PCR (NEBNext® library quantitation kit for Illumina, New England Biolabs, MA, USA). The sequencing was done on the Illumina platform using MiSeq Reagent V2 Micro (Illumina, CA, USA). After sequencing the FastQ file, the methylation level was determined on each gene site for each patient.

Methylation was carried out in cooperation with the HKG Epitherapeutics Limited laboratory (Hong Kong Science Park Shatin, Hong-Kong), bioinformatic analysis - in the Prof. Moshe Szyf laboratory (Canada) using the ChAMP package in R.

Integrated methylation analysis. The Student's t-test was used for each biomarker for paired comparison between groups. The sensitivity and specificity of the investigated DNA methylation markers' kit in BC were checked against the open DNA methylation databases (Illumina 450K): TCGA (cancer genome atlas), GSE40279, GSE61496, GSE76269, and GSE66836. Methylation data from international databases NCBI GEO and TCGA for BC and other cancers were also used for analysis and comparison. Correlation analysis with the definition of forward and reverse links between the promoter and enhancer methylation differences was carried out to determine functionally significant differences in DNA methylation in BC.

Ethical compliance during project implementation was ensured by obtaining informed consent from each patient for inclusion in this study. The study conduct followed the Research Protocol approved by the local ethics committee (LEC) of the Kazakh Institute of Oncology and Radiology (LEC minutes No. 13/17 of 28.09.2017).

Results and Discussion: The first stage of the search for BC detection markers included analyzing previously discovered facts [12, 15, 16]. The analysis showed that the methylation of CpG islets associated with specific genes (in BC, they most often include JAM3, C17orf64, MSC, and C7orf51) in BC samples was not typical for

other tumors and blood samples from healthy people. In BC, polygenic HKG-epiBreast-detect and spec markers showed a sensitivity of 0.94, a specificity of 0.91, an accuracy of 0.94, and an AUC of 0.95 (Figure 1).

88-97% of samples were identified as BC (Figure 1) as a result of the analysis using the weighted indicator of BC DNA methylation of the JAM3, C17orf64, MSC, and C7orf51 genes with the definition of their threshold value, and also when comparing with the "validation cohort" test data, including standardized Illumina 450K DNA beta-methylation values from the GSE101961, GSE60185, GSE60185, and GSE75067 databases, and the test data of 891 BC patients from the TCGA databases.

1A diagram shows the detection rate of HKG-epiBreast detect/spec markers in DNA methylation data of patients versus 16 other cancers. The potential of these markers in differentiating BC from other cancers was demonstrated using NCBI GEO and TCGA methylation data for BC and 16 other cancers. The ROC curve (Figure 1B) obtained by correlation analysis shows the specificity and sensitivity of the HKG-Breast-detect markers for BC detection using DNA methylation data from 4166 patients from the TCGA database.

We also analyzed 101 blood plasma samples of BC patients to discover the practicality of using the mentioned methylated genes as diagnostic markers of methylation for detecting BC in patients' blood plasma. The controls included a pool of plasma DNA and T-lymphocytes obtained from 50 healthy women of the relevant age.

The BC patients were divided by age, local status, tumor phenotype (by IHC), stage of the disease, data on relapses and progression of the disease, the presence of methylation.

The patients were aged 27 to 83 years (mean age – 52.02 ± 12.67 years). By race, 83 patients were Asian, and 18 were Caucasian. 40 (39.6%) patients had early BC at first disease manifestation (stages 0-IIA). Another 58 (57.5%) patients examined during the initial diagnosis and/or treatment had locally advanced BC, including 17 (16.8%) patients with metastases to various organs who were re-hospitalized (for the next course of treatment) after complex treatment for BC. The remaining 3 (2.9%) patients were hospitalized with primarily disseminated BC forms. Table 1 shows the patients' distribution by local status, indicating nodal, edematous-infiltrative, and ulcerative forms. Table 2 provides the BC patients' distribution by tu-

mor phenotype including luminal A and B (Her (-)) (56.4%), luminal B with Her2 overexpression (14.9%), triple-negative (10.9%), and Her2-enriched (17.8% of cases) types.

The number of methylated DNA molecules was visualized using a universal matrix visualization and analysis software Morpheus heat map (Broad Institute) [17].

In this analysis, all BC patients demonstrated methylation in the CpG islets associated with the JAM3, C17orf64, MSC, C7orf51 genes, and the CpG islet as-

sociated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434. The results reflected on the heat map (Figure 2) indicate a clear difference between BC samples and control samples. In the control group, the CpG islets of genes under study were not methylated. A combination of these areas' polygenic indicators allows detecting BC with high specificity and sensitivity, and accuracy above 90%. The JAM3 gene was also found to act as a tumor suppressor in another (colorectal) cancer [18].

Figure 1 – Illustration of the check for specificity (A) and sensitivity (B) of polygenic HKG-epiBreast-detect and spec markers for BC compared to other cancers in TCGA methylation data (n = 4166)

Table 1 – BC patients' distribution by local status

No. of patients, total	Nodal form	Edematous-infiltrative form	Necrotic ulcerative form
101	88 (87.2%)	11 (10.9%)	2 (1.9%)

Figure 2 shows the obtained plasma DNA methylation profiles in BC patients and healthy individuals.

The number of methylated DNA molecules was visualized using a universal matrix visualization and analysis software Morpheus heat map (Broad Institute) [17].

Table 2 – BC patients' distribution by tumor phenotype

Tumor phenotype	No. of patients (n=101)
Luminal A и B Her (-)	57 (56.4%)
Luminal type B with Her2 overexpression	15 (14.9%)
Her2-enriched	11 (10.9%)
Triple-negative type	18 (17.8%)

Then, we correlated the C7orf51, GC103, JAM3, MSC, and NYAP gene methylation level with the average age of patients at the time of diagnosis, stage of the disease, and tumor phenotype. A correlation analysis by

Spearman and the reliability of the difference between the parameters by the Kruskal-Wallis test demonstrated a significant positive relationship between the average age of the patients and the methylation level of the mentioned genes (Table 3). Those results were additionally confirmed by Pearson's correlation between the C7orf51, GC103, JAM3, MSC, and NYAP gene methylation level and age, as well as the stage of the disease. Among a large number of performed cor-

relations, the C7orf51 and GC103 gene methylation showed a positive correlation equal to 0.203 ($p < 0.05$); the JAM3 and MSC genes – 0.212 ($p < 0.05$); the JAM3 and NYAP genes – 0.02 ($p < 0.05$). The clinical significance of this correlation is not yet clear enough. However, we suggest that these genes' methylation is decisive in BC and can silence the tumor suppression genes. Other factors showed some positive correlation with no sufficient level of confidence (Table 3).

Figure 2 – Obtained plasma DNA methylation profiles in BC patients and healthy individuals: A – Methylation heat map for each of the five areas of biomarkers (color indicates the gene methylation level: red indicates methylation, blue – no methylation). The horizontal bar indicates BC samples and healthy tissue samples; B – the ROC curve shows the sensitivity and specificity close to 1.

The use of epigenetic techniques may seem secondary to the genetic profiling of the patient. However, standard genetic analysis reveals only the DNA nucleotide sequence. Improper methylation of a gene protecting cells from cancer will cause the gene's malfunction; the gene may fail to "turn on" and fulfill its protective function. In this view, epigenetic analysis is the only method of choice to detect potential cell disturbances. A study of the role of epigenetic techniques in diagnosing and treating malignancies, including BC, will uncover the processes occurring in the body during tumor development and help find effective approaches to early diagnosis and treatment of malignant neoplasms.

Thus, the assessment of tumor biomarkers, namely, methylation in the CpG islets associated with the JAM3, C17orf64, MSC, and C7orf51 genes and the CpG islet associated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434 in PBMC could be used as an alternative, minimally invasive objective method for early detection of BC.

Conclusions: The detected combinations of methylation in the CpG islets associated with the JAM3, C17orf64, MSC, and C7orf51 genes and the CpG islet associated with the intergenic region on chromosome 5, chr5: 77,208,034-77,329,434 can be found in both tumor-derived DNA and plasma of BC patients. These combinations can be differentiated from circulating

free DNA obtained from the plasma of healthy people. A specific tumor methylation profile against the background of a huge amount of DNA obtained from the

patient's blood or other normal tissue demonstrates the potential of plasma DNA methylation markers for non-invasive early BC detection.

Table 3 – Distribution of BC patients by groups according to clinical and phenotypic features and methylation

Clinical and pheno-typic features	All patients (n=101)	Patients with C7orf51 methylation (n=82)	Patients with GC103 methylation (n=70)	Patients with JAM3 methylation (n=30)	Patients with MSC methylation (n=29)	Patients with NYAP methylation (n=72)	Statistic method	Value	p-value
Average age, years [Min-Max]	52 [27-83]	52,6 [27-83]	53,3 [27-83]	51,7 [34-83]	53,7 [33-83]	51,3[27-83]	Корреляция Спирмена	0,2	0,049
Disease stages									
I	14 (13,9%)	12 (14,6%)	7 (10,0%)	2 (6,67%)	4 (13,8%)	10 (13,9%)	Корреляция Спирмена	0,02	0,88
II	61 (60,4%)	49 (59,9%)	43 (61,4%)	21 (70,0%)	18 (62,1%)	45 (62,5%)			
III	23 (22,8%)	18 (22,00%)	17 (24,3%)	6 (20,0%)	6 (20,7%)	16 (22,2%)			
IV	3 (2,9%)	3 (3,65%)	3 (4,3%)	1 (3,33%)	1 (3,4%)	1 (1,4%)			
Phenotype									
Lum A, B (Her (-))	57 (56,4%)	44 (53,7%)	37 (52,9%)	12 (40,0%)	16 (55,2%)	42 (58,3%)	Критерий Краскела-Уоллеса	12,9	0,07
Lum B+	15 (14,8%)	13 (15,8%)	12 (17,1%)	5 (16,7%)	4 (13,8%)	10 (13,9%)			
Her2+	11 (10,9%)	10 (12,2%)	9 (12,8%)	7 (23,3%)	5 (17,2%)	8 (11,1%)			
Triple-negative	18 (17,9%)	15 (18,3%)	12 (17,2%)	6 (20,0%)	4 (13,8%)	12 (16,7%)			

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ТҰЖЫРЫМ

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Эпигенетикалық маркерлер негізінде сүт безі қатерлі ісігін ерте диагностикалау әдісін әзірлеу

Өзектілігі: Бүгінгі таңда сүт безі қатерлі ісігі – қатерлі ісікті зерттеу жөніндегі халықаралық агенттіктің (МАИР-IARC) деректері бойынша, бүкіл әлем халқының арасында онкологиялық аурулар бойынша бірінші – екінші орында тұр [1], оның ішінде Қазақстанда [2] және әлемнің көптеген елдерінде әйелдердің осы қатерлі ісікпен сырқаттануы 1-орынды алады. Қазақстан Республикасында сүт безі қатерлі ісігі жыл сайын 27,2%-ға өсумен көш бастап келеді [2]. Қазақстан халқы арасында қатерлі аурулардан болатын өлім-жітім себептерінің құрылымында сүт безі қатерлі ісігі соңғы жылдары (2018-2019 жж.) 3-орынды иеленді. Осыған байланысты, сүт безі қатерлі ісігін диагностикалау проблемасының өткірлігі, атап айтқанда, аурудың ерте нысандарын анықтауды арттыру қажеттілігі сақталады. Қатерлі ісіктері бар науқастарды эпигенетикалық зерттеу нәтижелері эпигенетикалық биомаркерлерді ерте диагностикалық маркер ретінде, соның ішінде сүт безі қатерлі ісігін қолдануға мүмкіндік береді.

Зерттеудің мақсаты: Сүт безі қатерлі ісігі бар науқастардың перифериялық қанының моноклеарлы фракциясының жасушаларында ДНҚ метилизациясының профилін анықтау арқылы нақты диагностикалық маркерлерді іздеу.

Нәтижелері: JAM3, C17orf64, MSC, C7orf51 гендерімен байланысты CpG аралдарындағы және 5, chr5: 77,208,034-77,329,434 хромосомасының ішкі бөлігімен байланысты CpG аралындағы моноклеарлы қан фракциясының метилдену маркерлері сүт безі қатерлі ісігі бар қазақстандық популяция пациенттерінің плазма үлгілерінде анықталды және дені сау адамдарда болмады. Сүт безі қатерлі ісігін басқа қатерлі ісіктерден ажырату үшін осы биомаркерлерді қолдану мүмкіндігі ДНҚ метилизациясының ашық деректер базасындағы метилизация деректерімен салыстырғанда 0,91 және 0,94 сезімталдық деңгейімен көрсетілді (Illumina 450k үшін): TCGA (қатерлі ісік геномының атласы), GSE40279, GSE61496, GSE76269 және GSE66836.

Қорытынды: Сүт безі қатерлі ісігін ерте диагностикалау әдісінің ерекшелігі мен жоғары сезімталдығы пациенттердің перифериялық қанының моноклеарлы фракциясының жасушаларында ДНҚ метилизациясының профилін анықтау арқылы, атап айтқанда, JAM3, C17orf64, MSC, C7orf51 гендерімен байланысты CpG аралдарында және 5, chr5 хромосомасының ішкі аймағымен байланысты CpG аралында: 77,208,034-77,329,434 бұл әдісті сүт безі қатерлі ісігін скринингте қолдануға мүмкіндік береді.

Түйінді сөздер: сүт безі қатерлі ісігі (СБКІ), эпигенетика, ДНҚ метилденуі, клетки моноклеарной фракции крови, ранняя диагностика, биомаркер.

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Разработка метода ранней диагностики рака молочной железы на основе эпигенетических маркеров

Актуальность: По данным Международного агентства по исследованию рака (МАИР-IARC), на сегодняшний день рак молочной железы (РМЖ) занимает первое-второе места по онкозаболеваемости среди всего населения во всем мире [1], в том числе и в Казахстане [2]. В Республике Казахстан ежегодный прирост заболеваемости РМЖ составляет 26,6% и более. В структуре причин смертности от злокачественных заболеваний среди населения Казахстана, РМЖ занимает в последние годы 3 место, на его долю приходится 8,7-8,1% по данным 2018-2019 гг. Сохраняется острота проблемы ранней диагностики РМЖ. Результаты эпигенетических исследований больных со злокачественными опухолями позволяют предложить эпигенетические биомаркеры для использования в качестве ранних диагностических маркеров, в том числе и для РМЖ.

Цель исследования: Определение специфичных диагностических маркеров путем установления профиля метилирования ДНК в клетках моноклеарной фракции периферической крови больных раком молочной железы

Результаты: Маркеры метилирования моноклеарной фракции крови в островках CpG, связанные с генами JAM3, C17orf64, MSC, C7orf51 и в островке CpG, связанного с внутригенным участком хромосомы 5, chr5: 77,208,034-77,329,434, были выявлены в образцах плазмы пациентов казахстанской популяции с РМЖ и отсутствовали у здоровых людей. Возможность применения этих биомаркеров для дифференциации РМЖ от других видов рака была продемонстрирована с уровнем специфичности 0,91 и чувствительности 0,94 при сравнении с данными метилирования открытых баз данных метилирования ДНК (для Illumina 450K): TCGA (атлас ракового генома), GSE40279, GSE61496, GSE76269 и GSE66836.

Заключение: Специфичность и высокая чувствительность способа ранней диагностики РМЖ путем определения профиля метилирования ДНК в клетках моноклеарной фракции периферической крови пациенток, а именно в островках CpG, связанных с генами JAM3, C17orf64, MSC, C7orf51 и в островке CpG, связанного с внутригенным участком хромосомы 5, chr5: 77,208,034-77,329,434, позволяют использовать данный способ при скрининге РМЖ.

Ключевые слова: рак молочной железы (РМЖ), эпигенетика, метилирование ДНК, клетки моноклеарной фракции крови, ранняя диагностика, биомаркер.