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The role of epigenetic research in diagnostics and treatment of breast cancer

Relevance: *Genetic mutations play an essential role in the development of malignant tumors; still, the part of epigenetic processes can also be significant. Epigenetic changes are not inherited and do not violate the DNA nucleotide sequence. However, they can critically change their accessibility for the transcriptional apparatus of the cell by chemical modification of specific chromosomal loci and associated genes. Acetylation and methylation of histones or DNA are examples of epigenetic changes. Epigenetic activation/deactivation of genes is the basis for the differentiation of all types of somatic cells. A change in the specific "epigenetic labeling" of a cell of a particular type causes cell de-differentiation or results in changing its phenotype (transdifferentiation). Given the scale of epigenetic regulation, it is logical that any violations of this process can lead to pathologies, including cancer.*

This paper reviews the modern approaches and methods of study of the molecular genetic basis for the development of malignant tumors and epigenetic mechanisms, in particular, DNA methylation in breast cancer.

Purpose of the study *is to review and analyze the existing molecular genetic methods for studying DNA methylation in breast cancer.*

Results: *DNA methylation is a critical mechanism of epigenetic modification, which is involved in gene expression programming and can contribute to the development of cancer, including breast cancer. Methylation of CpG islands of DNA methyltransferase, which is usually reversible, modifies the transcriptional activity of main proliferation genes or transcription factors involved in cell growth suppression or stimulation.*

Many genes can serve as biomarkers for early detection of breast cancer. For example, the researchers have detected hypermethylation of the DOK7 gene promoter portion, which is one of the diagnostic specific epigenetic markers of breast cancer. The study results we have analyzed show that combined epigenetic therapy leads to a synergistic antitumor response in patients with breast cancer [1-9].

Conclusion: *The currently available diagnostic and treatment methods aimed at aberrant methylation with DNA methyltransferase inhibitors trigger the repeated expression of "silent" genes and make it possible to increase the effectiveness of treatment. Aberrant methylation found in gene promoters is a sign of oncological transformation that can serve as a non-invasive biomarker in body fluids (blood, plasma) for early detection of breast cancer. However, there is currently no unique biomarker that would have sufficient specificity and sensitivity for use in therapy.*

Therefore, for a particular therapeutic case, a panel of several genes is required.

Thus, the methods of epigenetics were found to have vast diagnostic and therapeutic potential.

Keywords: *molecular and genetic methods, epigenetics, DNA methylation, breast cancer, biomarker.*

Introduction. Breast cancer (BC) is leading in the structure of cancer diseases. WHO reports more than 1.38 million new cases of BC every year in the world, at the death rate, is about 460 thousand. In the Republic of Kazakhstan, BC also ranks first in incidence. 4653 cases of BC registered in 2016 made 26.1 per 100,000 populations of both sexes. 1-year mortality was 5.4%, and 5-year survival was 57.8% [1]. Despite developed screening programs and high awareness of BC, there are still a high proportion of female patients who are admitted with grade III-IV of the disease. Therefore the survival rates remain extremely low. It maintains the relevance of early diagnosis of primary cancer and its recurrence, as well as the selection of effective therapy.

There is a growing number of papers [2–17] devoted to the study of molecular biological factors that regulate the mechanisms controlling cell division and death, as well as the maintenance of genetic stability and study of epigenetic mechanisms of cancer.

Epigenetic changes are changes in gene expression which come without any disturbances in the DNA sequence. They are important critical factors in cancer development and disease prognosis caused by the development of genetic and phenotypic instability [2]. Modifications can occur at both the genetic and epigenetic levels.

For example, a mutation in the tumor suppressor gene (TSG) indicates the accumulated DNA damage. In the absence of DNA repair, such cell becomes more prone to cancer development. Epigenetic disorders in the expression of main genes, including TSG, were found to play an essential role in carcinogenesis in general, and especially in the development of BC [3-5]. The increase in epigenetic disorders makes cancer cells more aggressive and changes their behavior from invasion in surrounding tissues to dissemination in lymphatic and blood vessels. This ultimately leads to the death of the patient if untreated.

Such epigenetic change as aberrant DNA methylation does not include changes in the DNA sequence; however, this covalent chemical modification of DNA has a significant influence on the whole gene expression. This altered gene expression leads to many accumulated changes which promote oncogenesis [6]. The use of epigenetic

methods, at first glance, seems to be secondary in comparison with the determination of the patient's genetic profile. However, standard genetic analysis reveals only the DNA nucleotide sequence that may not be disrupted. However, if the gene that protects the cell from cancer is methylated incorrectly, the gene can become malfunction and fail to perform its protective function.

In this case, epigenetic analysis is the only way to detect potential cell violations. An important difference between genetic and epigenetic changes is that drugs are efficient against epigenetic changes but absolutely helpless against genetic mutations [2].

Understanding the role of epigenetic methods in diagnostic and treatment of malignant tumors, in particular, BC, will provide a better view on the processes occurring in the body during tumor development and allow finding efficient approaches to their early diagnostics and treatment.

The purpose of the study was to review and analyze the existing molecular genetic methods for studying DNA methylation in BC.

***Materials and methods*:** The published sources on the topic "DNA methylation methods for diagnosis and therapy, in particular, early diagnostics and therapy of BC" were analyzed, including fundamental works of scientists, articles in scientific periodicals, materials of conferences and symposiums. The search in electronic databases was done by the following keywords: "epigenetics," "DNA methylation," "epigenetics in breast cancer diagnosis."

Results and discussion:

The use of DNA methylation as a biomarker in the early detection of *И*C.

Currently, histological verification and determination of invasion grade and tumor phenotype make the gold standard of BC diagnostics. These diagnostic methods require a biopsy of tumor material. Biomarkers of high sensitivity and specificity can be found in tissues and fluids of the patient's body. Since changes in the DNA methylation pattern are one of the earliest modifications in cancer development, these changes can serve as a biomarker for early detection of BC [7–8] as they make testing more objective.

Various methods to assess DNA methylation include bisulfite conversion, which is performed using a panel of selected genes and primers for amplifying CpG islands specific for Real-time-PCR methylation [3]. The general genomic approach with high-throughput sequencing utilizes bisulfite-converted DNA obtained from various non-invasive biological sources such as whole blood, serum, and plasma [3].

Several promising epigenetic markers for early BC detection are based on peripheral blood genomic analysis. For example, Yan et al. have found that the CpG islands of hyaluronoglucosaminidase 2 (HYAL2) were significantly hypomethylated in the peripheral blood of BC patients compared with the control group.

The level of blood HYAL2 methylation also acts as an early predictor of BC compared with the control group, with 64% sensitivity and 90% specificity [8]. At that, HYLA2 locus is hypermethylated specifically in BC tissue but hypomethylated in the blood, which indicates the same risk of BC. Therefore, HYAL2 hypomethylation in the blood can also be an early non-invasive peripheral biomarker.

Another similar study on the plasma of patients with BC and healthy women has revealed hypermethylation of kinesin 1A family (KIF1A) promoter in BC. The researchers concluded that high KIF1A promoter methylation in plasma could also be an early biomarker of BC [3]. The analysis of DNA methylation in twins has shown that the dock protein 7 (DOK7) promoter was hypermethylated in the blood of patients compared to their twins. Such hypermethylation was present several years before the diagnosis.

Consequently, DOK7 promoter methylation level could potentially be a biomarker for early detection of BC [9]. The status of DNA methylation inside and outside the CpG islands of a certain number of genes has shown that some of them were associated with the risk of BC. Kloten et al. have evaluated the methylation status of the TSG group (secreted by SFRP1, SFRP2, SFRP5 associated with proteins), the heavy chain family of the alpha trypsin inhibitor, member 5 (ITIH5), a WNT inhibitory factor 1 (WIF1), Dickkopf inhibitor WNT signaling pathway 3 (DKK3), and RASSF1A) [10] in circulating tumor DNA. A particularly important finding was that the DKK3 and ITIH5 CpG islands were unmethylated in women with benign breast pathologies and significantly hypermethylated in women with BC. The researchers hypothesized that promoter methylation of DKK3 and ITIH5 in blood could be used as a biomarker, mainly in patients with dense breast tissue, while RASSF1A methylation of CpG islets was not a good biomarker taking into account its low rate in healthy women.

Brennan's et al. [11] have studied the DNA methylation status outside the CpG of island ATM serine/threonine kinase (ATM) clusters and the repetitive elements of a long interspersed nuclear element 1 (LINE1) in leukocytes in a large group of women with and without BC. They observed higher methylation of the ATMmvp2a locus in BC patients compared with the control group and concluded that the locus could be used as a biomarker for the risk of BC. They also found that the association of ATM methylation with the risk of BC was more reliable in young women and that the biomarker remained stable for at least six years [11].

Kuchiba et al. found that the global DNA methylation of peripheral blood leukocytes was below the norm in patients with BC and could be a potential biomarker for the risk of BC [12].

Data analysis has also revealed about 10,000 sites in T-cells that correlated with BC progression. The scientists compiled a list of 89 CG sites highly correlated ($p < 0.01$, $r > 0.7$, $r < -0.7$) with BC progression. The vast majority of those hypomethylated sites were directly related to the genes responsible for immune system function [13].

Thus, the assessment of tumor biomarkers could be used as an alternative minimally invasive objective method of early detection of BC.

Treatment of BC adjusted for the regulation of methylation. Detection and treatment of BC at an early stage (stages I and II) improves 5-years survival (> 93%) compared to the late detection of metastatic cancer (stage

IV – 22%) [14]. Numerous TSGs (for example, genes of DNA repair, apoptosis, hormone receptors, cell cycle and genes of transcription factors) were found to be differentially methylated in BC and, accordingly, could serve as good therapeutic targets [15]. Therefore, the treatment of BC by regulating proteins involved in methylation processes was found to be a promising method of treatment.

Candidate treatment methods of BC include regulation of methylation activity using DNA methyltransferase (DNMT) inhibitors. Lower DNMT activity inhibits tumor growth due to increased expression of "silent" genes, such as TSG, estrogen alpha receptor genes, E-cadherin, and SFRP. Cytidine analogs, such as decitabine (5-aza-2'-deoxycytidine) and 5-azacytidine, act as DNMT inhibitors and, thus, can reactivate the expression of main genes by depleting DNMT1 [16].

Both of these analogs have been approved by the US Food and Drug Administration (FDA) for the treatment of the myelodysplastic syndrome. These residues of the DNMT inhibitor are incorporated into the DNA during the S-phase of the replication process and establish irreversible bonds with DNA-methyltransferase enzymes to prevent their action [17]. Other studies [18] also reported an increase in the effectiveness of treating BC with a similar approach. Studies have been conducted in vivo to evaluate the effect of the above compounds on solid breast tumors. However, in addition to their poor stability and lack of specificity for cancer cells, these drugs are quickly inactivated by cytidine deaminase.

Consequently, these drugs have serious limitations for treating advanced solid tumors, including BC. In addition, these agents can activate a panel of prometastatic genes in addition to the activation of tumor suppressor genes, thus increasing metastasis. The question that has to be answered is how to target tumor suppressor genes and block the growth of cancer with DNA demethylation drugs while avoiding activation of prometastatic genes and preventing cancer metastasis [19].

These disadvantages have led to the development of new DNMT inhibitors, namely, zebularine, SGI-110, and NPEOC-DAC, which are more selective for cancer cells and demonstrate a higher resistance to deamination. Zebularine has a potent inhibitory effect on both DNMT and cytidine - deaminase.

Zebularine has a potent inhibitory effect on both DNMT and cytidine deaminase. It was shown in vitro that Zebularine in combination with decitabine has a significant inhibitory effect on cell proliferation and colony formation in the MDA-MB-231 breast cancer cell line by inducing the expression of mRNA estrogen receptor alpha and progesterone [20]. It has also been shown that this drug inhibits the growth of breast tumor cells in vivo, causing necrosis and apoptosis of tumor cells with early onset in transgenic mice that develop mammary tumors [21]. Although zebularine initially showed promising effects associated with its high selectivity against cancer cells, its toxicity makes this drug less attractive for the treatment of breast cancer patients. SGI-110 is a modified dinucleotide that exhibits increased resistance to cytidine-deaminase and an increased half-life period compared with decitabine and 5-azacytidine [22]. This short oligonucleotide may be provided to ensure efficient delivery of the nucleotide drug and protection against deamination. NPEOC-DAC is a metabolic precursor of the decitabine molecule and has a dose-dependent depressive effect on DNA methylation [23].

Some other natural compounds containing specific molecules, such as anthocyanins and polyphenols, were found to have anti-DNMT activity [24]. The DNMT1 knockdown, with the help of small interference RNA has also led to promising results in HCT116 colon cancer cells [25].

Since the side effects of DNMT inhibition include concomitant activation of both TSG and proto-oncogenes, some studies have evaluated the impact of a combination of cytidine analogs with chemotherapeutic agents, immunotherapy or specific genes knockdown, such as methyl-CpG (MBD2) protein 2 and lysine (K) -specific demethylase 1B (KDM1B/LSD2) [15]. Vijayaraghavalu et al. noted a significant increase in the effectiveness of doxorubicin treatment in MCF-7, MDA-MB-231, and BT-459 cells in combination with decitabine [26]. Such dual treatment caused a cessation of the cell cycle phase for more than 90% of the cells and resulted in the restoration of sensitivity to doxorubicin by increasing the expression of the p21 proto-oncogene. In turn, it allowed overcoming the drug resistance of the breast cells. In another study, Wrangle et al. have evaluated the sequential combination of 5-azacytidine and immunotherapy and shown that combined epigenetic therapy using a DNA methyltransferase inhibitor and a programmed death blockade led to a synergistic antitumor response, also in patients with non-small-cell lung cancer [27].

On the one hand, MBD2 suppresses methylated genes, but on the other, it is involved in the activation of gene expression due to its ability to interact with gene promoters. A recent study has shown that the introduction of a methyltransferase inhibitor (5-azacytidine) combined with suppressing MBD2 expression using RNA interference technology activates apoptosis and decreases cell growth, as well as inactivates the invasive and metastatic processes in BC cells. Simultaneous inhibition of depletion of MBD2 (methylated DNA binding protein 2) and DNA methyltransferase (DNMT) in BC cells leads to a combined in vitro and in vivo effect: it enhances the delay in tumor growth while inhibiting the invasiveness caused by 5-azaCdR. The combined treatment of MBD2 and 5-azaCdR depletion suppresses and strengthens various gene networks induced only by DNMT inhibition. This indicates the potentially new approach to targeting DNA methylation mechanism by a combination of MBD2 and DNMT inhibitors [28]. MBD2 depletion counteracts the proto-oncogenes activation as a result of hypomethylation therapy. The same strategy, including DNA methyltransferase inhibition and LSD2 knockdown, is also efficient in inhibiting MDA-MB-231 and MCF-7 BC cell growth. Such therapy enhances the expression of epigenetically silenced genes such as the genes encoding the progesterone receptor and the alpha-receptor estrogen [29].

Mahmood et al. have shown that treatment using SAM (universal methyl-S-Adenosyl methionine donor) significantly decreases proliferation, invasion, and migration de-

pendent on the dose and independent of growth fixation and increases apoptosis in vitro. The results have been reproduced in vivo: oral administration of SAM reduced tumor volume and metastasis in an MDA-MB-231 xenograft model labeled with a green fluorescent protein (GFP).

Gene expression analysis has confirmed the ability of SAM to reduce the expression of several main genes involved in cancer progression and metastasis, both in cell lines and in BC xenografts. The results of this study provide good evidence for evaluating the therapeutic potential of methylating agents, such as SAM, for reducing the morbidity and mortality from BC [31].

Conclusion: In the past decade, several gene expression signatures were established to characterize and subtype breast tumors. However, at present, methylation is considered the leading player which regulates gene expression. Unlike RNA transcription profiles, which illustrate the transcriptional activity at a specific moment, DNA methylation status is a more stable and long-lasting marker of the molecular state and susceptibility of cells to cancer.

Though many models of metastatic processes have been proposed, recent studies show the metastatic capacity of breast tumors to be an inherent feature of the host's genetic background. Epigenetic changes are assumed to occur at the early stages of breast carcinogenesis before the metastatic process, and, therefore, the methylation profile, to a certain extent, reflects the genetic background of individuals.

All these observations confirm the potential significance of the evaluation of the methylation profile in biological fluids for accurate risk determination in women prone to or affected by BC. Considering that circulating cell-free plasma DNA contains tumor-specific mutations and DNA methylation patterns associated with the disease, identification of new biomarkers being the precursors of potential susceptibility to cancer or aggressiveness in such DNA will be a huge achievement in predictive medicine for women at high risk of BC.

Minimally invasive testing, such as screening for epigenetic changes in the blood, is a fairly convenient and objective technique. However, mainly due to the limited number of affected and matched control DNA samples in the studied cohorts, no specific methylation biomarker has yet been confirmed for clinical use.

Combined studies of the entire epigenome could contribute to the creation of a whole group of BC biomarkers to improve the diagnostics and early detection of BC in women. Although some DNMT inhibitors are reported to increase the effectiveness of standard chemotherapy only for certain types of cancer, further studies of the effect of demethylation agents in vivo on various solid tumors are quite reasonable. It should not be forgotten that DNMT inhibitors increase the rate of cell division and proliferative activity. Therefore, repeated treatment is required to increase their antitumor efficacy [28]. Beyond that, such factors as toxicity, the lack of specificity, low stability, and the simultaneous activation of proto-oncogenes impede the development of new inhibitors. However, the combined use of these DNMT inhibitors with other types of agents, such as chemotherapeutic drugs and RNA interference, gives promising results.

Thus, epigenetic methods in combination with wider access to minimally invasive biological material (blood, plasma) are crucial for a deeper understanding of the biology of BC and the development of new methods for early diagnostics and personalized therapy to improve the prospects for patients with BC.

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