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DNA Methylation as Epigenetic Biomarker of Breast Cancer

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Abstract: DNA methylation plays a crucial role in regulating gene expression and maintaining cellular function. In recent years, researchers have become increasingly interested in its involvement in carcinogenesis, particularly in breast cancer (BC). Changes in DNA methylation patterns have been implicated in the progression of cancer, including BC. Global hypomethylation, which refers to a decrease in overall methylation levels, can lead to the inactivation of oncogenes. On the other hand, hypermethylation, an increase in methylation levels, has been associated with the inactivation of tumor suppressor genes (TSGs). It is worth noting that the targeted alteration of TSGs in eradicated BC tissues/liquid biopsy, plasma, and peripheral blood biospecimens could potentially impact the sensitivity of BC patients to chemotherapy, hormone therapy, and immunotherapy. This highlights the potential of identifying specific TSG targets through DNA methylation analysis, opening possibilities for novel epigenetics drug (epi-drug) therapies. At present, there is a lack of non-invasive biomarkers that can provide early detection of BC. Environmental exposures can induce changes in DNA methylation, which in turn may contribute to the development of BC. Additionally, DNA methylation alterations have been observed in triple-negative BC (TNBC), which is the most invasive subtype of BC. Consequently, the detection of DNA methylation changes in liquid-based assays for patients with BC could aid in earlier identification of the disease and more accurate prediction of epi-drug therapy outcomes. In summary, DNA methylation alterations play a significant role in BC, and studying these changes offers potential paths for improving early detection and developing targeted epi-drug therapies.

Key words: Breast cancer · Epigenetics · DNA methylation · Hypermethylation · Hypomethylation · Prognostic biomarker · Diagnostic biomarker · Epi-drug

INTRODUCTION

The concept of epigenetics was initially introduced by Waddington in 1942 [1], emphasizing the influence of environmental factors on traits through gene-environment interactions. Waddington's idea of genes interacting with the environment to shape phenotypes is significant in developmental biology. Epigenetics explores how an organism's traits are determined by a combination of its genome and environmental influences. Environmental factors play a role in promoting organismal development, understanding the structural aspects of genetic material, and elucidating the mechanisms that regulate modifications such as DNA methylation, histone modifications, and small non-coding RNAs [2].

Recent advancements in epigenetics have shed light on the modifications of DNA and/or proteins that can transmit information to the next generation without altering the DNA sequence. Six years after Waddington's introduction of epigenetics, DNA methylation was recognized as an epigenetic biomarker [3]. Currently, researchers are focusing on understanding DNA

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methylation modifications to gain deeper insights into epigenetics. DNA methylation has been found to be involved in gene inactivation, particularly in cancer cells, across various cell types [4].

Early detection of BC is crucial for effective disease management, yet there are currently no established epigenetic biomarkers for BC screening or diagnosis. BC is the second most common type of cancer, accounting for 14% of all cancer cases, following lung cancer at 14.6% [5]. It is the leading cause of cancer-related deaths among women worldwide. Most BC cases (approximately 40%) occur after the age of 40, while 6.6% arise before this age [6].

The prognosis of BC is closely tied to early diagnosis, which is typically achieved through mammography [7]. Biomarkers such as CA15-3 and CA27-29 are used for monitoring disease progression and treatment efficacy. However, there is a need for reliable epigenetic biomarkers that can aid in BC screening and diagnosis [7, 8].

The majority of BC cases are considered sporadic and are associated with environmental factors [9]. However, around 20% of cases have a familial component, with 5-10% being attributed to autosomal dominant mutations that increase the risk of both breast and ovarian cancer and approximately 25% of BC cases involve germline mutations in major susceptibility genes, such as DNA damage responsible breast cancer 1 and 2 (*BRCA1* & *BRCA2*), tumor protein p53 (*TP53*), checkpoint kinase 2, ATM serine/threonine kinase (*ATM*), and phosphatase and tension homolog (*PTEN*) [10].

BC exhibits heterogeneity at both the molecular and histological levels. It is a complex process involving genetic alterations, activation of proto-oncogenes, and inactivation of TSGs. The interplay between genetic and environmental risk factors is regulated by epigenetic mechanisms, particularly DNA methylation modifications, which can lead to dysregulation of biochemical pathways associated with BC [11].

Differences in DNA methylation profiles between BC patients and healthy controls can provide insights into the molecular mechanisms underlying BC development and may lead to the discovery of new prognostic and diagnostic biomarkers [12]. For instance, hypermethylation and global hypomethylation of 5` — C — phosphate — G — 3`(CpG) islands are early molecular changes observed in BC patients, suggesting their potential use in guiding early treatment decisions [13]. Furthermore, DNA methylation alterations have been observed during the transition from healthy breast tissue to ductal carcinoma in situ (DCIS), although the changes from DCIS to invasive ductal breast carcinoma (IDC) are less pronounced. So, these DNA methylation changes occur in the early stages of BC and can serve as biomarkers for early diagnosis [14].

DNA methylation profiles can be evaluated not only in tumor tissue and peripheral blood biospecimens but also by extracting circulating tumor DNA (ctDNA) from plasma using liquid-based methods. This approach enables the assessment of primary lesions and metastases, providing valuable information for cancer detection, residual disease monitoring, and individualized therapy strategies [15]. ctDNA analysis offers advantages over tissue biopsy, as it overcomes challenges related to tumor location and sample size. It simplifies the detection of DNA methylation and provides valuable data for clinical decision-making [16].

Focus on the Basic Mechanism of DNA Methylation:

DNA methylation can be influenced by various factors, including stress, aging, high alcohol consumption, physical activity, air pollution, and depression. These factors can impact biological pathways such as X chromosome inactivation, genome reprogramming and differentiation, genomic imprinting, development and survival, and genetic molecular alterations [17, 18]. All these factors, either individually or in combination, can contribute to the development of breast cancer (BC) [19].

The process of DNA methylation involves the addition of a methyl group to the cytosine base of CpG dinucleotides, which is mediated by enzymes called DNA methyltransferases (DNMTs) (Fig. 1). Methylated DNA generally leads to transcriptional repression of specific genes. Hypomethylation, on the other hand, can increase gene expression and activate specific genes. Methylation of the promoter region can result in reduced gene expression by preventing activator proteins from binding to the chromodomain, which recognizes methyl groups. In diseases, promoters and CpG islands associated with actively expressed genes are typically hypomethylated compared to healthy individuals, and this may also occur in cancer patients [20]. A hypermethylation of CpG islands, especially which located in the initial region of exons, is associated with genomic instability, and can lead to the silencing of genes. Different types of tumours can arise due to CpG island methylation throughout the genome or somatic mutations in regulatory genes [21].

DNA methylation alterations are observed in BC tissue and can be influenced by various factors, including aging [22]. Generally, DNA hypermethylation within

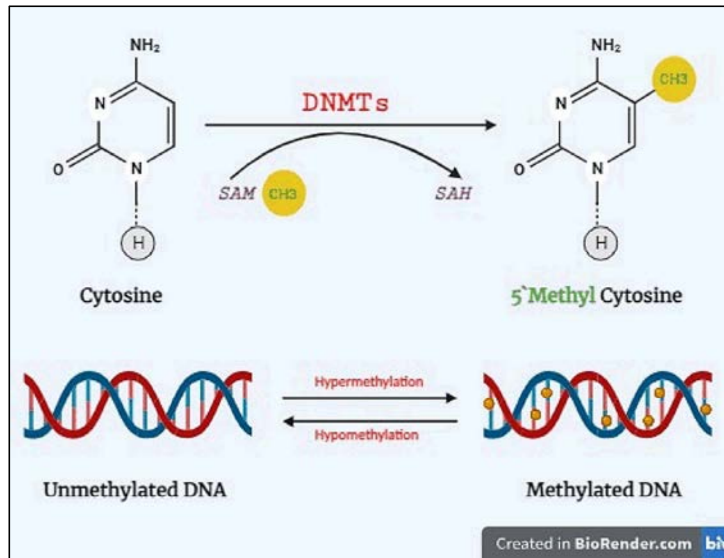

Fig. 1: The process of DNA methylation. DNMTs enzymes were responsible for catalyzing the transfer of the methyl group. S-Adenosyl methionine (SAM), a common methyl donor molecule in biological processes, provides the methyl group for this reaction. The methyl group is added specifically to the carbon 5 position of the cytosine ring, leading to the formation of 5-methylcytosine.

regulatory regions such as promoters and CpG islands can inhibit gene transcription, causing the affected gene to act as a tumour suppressor. However, the relationship between intragenic (exons and introns) and intergenic (enhancers) DNA methylation and gene expression is also gaining importance in understanding its impact on cancer risk. Further investigation is needed to understand the effects of DNA methylation in the promoter region versus the gene body [23].

In normal cells, actively transcribing chromatin is generally hypomethylated, while methylated chromatin forms small structures that physically block the processes of RNA Pol II [24]. DNA methylation is known to play a role in stabilizing the inactive chromatin structure and suppressing gene transcription [25], thereby contributing to important biological processes such as X-chromosome inactivation, differentiation, and genomic imprinting [26, 27].

Cytosine is the primary target for DNA methylation. CpG dinucleotides, which are commonly found in gene promoter regions, contain the highest levels of 5'-methylcytosine in mammalian DNA. Approximately 72% of human gene promoters have a high CpG content, while 28% have a low CpG content, similar to the overall genome. CpG sites are distributed approximately once per 80 dinucleotides in 98% of the human genome, but CpG islands, which constitute 1-2% of the genome, are relatively sparse [28].

DNA methylation and demethylation are essential regulatory features of the genome. However, abnormal DNA methylation can significantly disrupt important functions. Hypermethylation of promoter regions in TSGs and DNA repair genes, including (*BRCA1, p15, p16, p53, p57, and* solute Carrier Family 5 Member 8 *SLC5A8*) has been associated with the development of various cancers, including BC [29-32]. On the other hand, DNA hypomethylation can lead to genomic instability, which is implicated in breast and other types of carcinomas. For example, studies have shown that leukocyte genomic DNA hypomethylation is associated with an increased risk of bladder cancer [33]. Hypermethylation primarily occurs at CpG islands in the promoter regions of genes, while hypomethylation is linked to repetitive DNA sequences, such as long interspersed nuclear elements [34]. Global hypomethylation can lead to the activation of transposable elements (TEs), causing them to transcribe across both sense and antisense strands [35].

Methylation of Genes Associated with BC: DNA methylation has been associated with wide range of functions, including development and gene transcription regulation, as well as genomic imprinting. Recent studies have emphasized the significance of DNA methylation as an epigenetic biomarker for distinguishing between normal and BC in humans. In normal cells, CpG islands located before gene promoters are typically unmethylated,

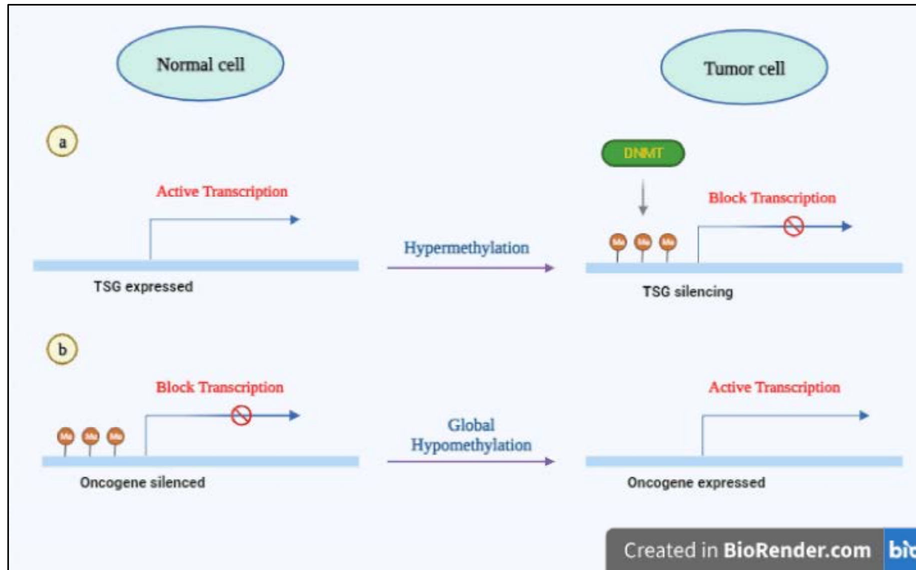

Fig. 2: The relationship between DNA methylation and the expression of tumor suppressor genes (TSGs) and oncogenes in normal cells and tumor cells.
(a) In normal cells, TSG are expressed and typically exist in a demethylated state. This means that the DNA regions associated with these genes have low levels of methylation. The demethylation allows for TSG expression, which plays a critical role in regulating cell growth and preventing the development of cancer. On the other hand, oncogenes, which have the potential to drive uncontrolled cell growth, are generally unexpressed in normal cells and exhibit higher levels of DNA methylation.
(b) In tumor cells, there is a shift in the DNA methylation pattern compared to normal cells. TSG can undergo de novo methylation, a process where DNMTs add methyl groups to previously unmethylated DNA regions associated with these genes. This DNA hypermethylation leads to the silencing or inhibition of TSG expression. Interestingly, tumour cells often exhibit global hypomethylation, which means a decrease in DNA methylation levels across the genome. This global hypomethylation can contribute to the activation and increased transcription of oncogenes.

while CpG dinucleotides across the genome may be methylated. However, in cancer cells, CpG islands preceding TSG promoters are frequently hypermethylated, while CpG methylation of oncogene promoter regions and repetitive sequences is often hypomethylated (Fig. 2). Hypermethylation of gene promoters can lead to gene silencing, which can be detrimental when genes involved in cell cycle regulation are silenced, allowing cells to divide and replicate uncontrollably, leading to cancer. Numerous hypermethylated genes associated with BC have been identified, including genes regulated DNA repair (e.g., *ras* association domain family member 1 (*RASSF1A*) and *BRCA1),* also those involved in cell cycle regulation (*e.g.,* cyclin D2 (*CCND2*), cyclin dependent kinase inhibitor 2A (*CDKN2A*)) (Table 1), and cell adhesion (e.g., *E- cadherin* (Table 4). Also, several proteins associated with BC have been revealed related with cell signalling (e.g., estrogen receptor (ER)), and more mentioned in (Table 2).

BRCA1 is located on chromosome 17q21 [36]. Its protein product is 1863 amino acid (NCBI amino acid sequence), and it is well-known for its link to tumour suppressor activity in breast and ovarian cancers. There is ample evidence that *BRCA1* performs various functions in genomic safety and stability. It plays an important role in the response to DNA damage, the cell cycle checkpoint, and the repair of double-strand breaks. There is proof that part of its repair and checkpoint response function is associated with tumour-suppressing behaviour [37]. One-half of all hereditary breast carcinomas are caused by *BRCA1* gene mutations, and an investigation of hypermethylation events preceding the *BRCA1* promoter discovered methylation in 11% of sporadic BC cases [38]. Another BC study revealed that the *BRCA1* promoter is methylation in 13% of unselected primary BC and hypermethylation events preceding the *BRCA1* promoter have been observed in a subset of sporadic BC cases. Loss of heterozygosity (LOH) for

Table 1: Several methylated genes in BC.

Gene	Gene description	Ref.
AKT1	Methylation of the *AKT1* gene has been linked to BC and can impact its expression level. The presence of the human epidermal growth factor receptor 2 (HER2) protein has also been associated with *AKT1* expression.	[56]
BRCA1	*BRCA1* is a TSG that plays a crucial role in maintaining genomic stability. Methylation of the *BRCA1* promoter region can influence gene expression and lead to the loss of tumor suppressor activity.	[57]
CDKN2A	In patients with malignant tumours, it is methylated, whereas in those with benign breast disease, it is unmethylated. This gene has been associated to distant BC metastasis.	[58]
E2F4	A low transcription factor that promotes tumour development is a possibility. Methylation of the gene can boost gene expression and speed up tumour development.	[59]
ESR1	Aberrant hypermethylation of the *ESR1* gene has been detected in BC cells, and it may serve as a novel biomarker for the disease.	[60]
FHIT	The *FHIT* gene, which is highly expressed in normal tissues, exhibits methylation in 31% of primary BC patients. Methylation of *FHIT* can alter its expression levels in patients with sporadic ductal carcinoma.	[61]
HSD17B4	Methylation of the *HSD17B4* gene has been identified as an independent predictor of pathological complete response in certain studies. Trastuzumab treatment may be ineffective if the gene is not methylated in BC individuals, but lapatinib treatment can be successful.	[62]
Hox	Methylation of *Hox* genes is associated with increased expression of ER and progesterone receptor (PR). Methylation of the *HoxD13* gene is related to larger breast tumor size and poor clinical response to therapy.	[63]
MDGI	The *MDGI* gene is expressed at low levels in BC tissues. Methylation of the *MDGI* gene's promoter region in BC patients may have a minimal effect on surgery outcome but a more noticeable impact on the effectiveness of tamoxifen treatment.	[64]
NBPF1	BC has hypermethylation in the promoter region of this gene. Patients' methylation *NBPF1* in serum or plasma may become a possible tumour biomarker for identifying BC.	[65]
PITX2	*PITX2* has been identified as a prognostic biomarker specifically for individuals with progesterone receptor-positive BC. Methylation of the *PITX2* gene is associated with decreased survival and an increased risk of distant metastases. However, if the gene is methylated, there is a lower probability of distant metastasis recurrence, which may eliminate the need for additional treatment.	[66]
RASSF1A	This gene is methylated in 33.3 % of patients with sporadic BC.	[67]
Soxl7	The *Soxl7* gene has been associated with breast tumor diameter and lymphatic metastasis. However, this gene has been found to be unmethylated in normal breast tissue and serum.	[68]
TIMP-3	The methylation of the *TIMP-3* gene is specifically observed in BC cells and not in other tissues. The extent of methylation is associated with the malignancy of BC.	[69]

Table 2: Several oncogene proteins related to BC.

Protein	Protein description	Ref.
CA125	CA125 is a predictive biomarker for ovarian and breast carcinoma. It plays an interactive role in disease processes.	[70]
CA19-9	The expression of this protein has been associated to therapy response and BC survival.	[71]
CA153	In BC, the breakdown of the cytoskeleton and increased protease and salivary enzyme activity lead to the release of the CA153 saccharide antigen from cancer cell membranes into the blood.	[72]
CEA	CEA is an acidic glycoprotein that contains a human embryonic antigen determinant. It is a broad-spectrum tumor biomarker found in various tumors, including BC, lung cancer, and other malignancies.	[73]
CypB	CypB protein is more abundant in malignant cells compared to non-cancerous tissues. Functional studies suggest that a small amount of CypB suppresses tumor cell growth, proliferation, and migration.	[74]
ER	Estrogen receptor (ER) is critical in the pathophysiology of BC and can be used as an indicator to guide pharmacotherapy for breast cancer patients.	[75]
HER2	Human epidermal growth factor receptor 2 (HER2) is a relevant therapeutic and prognostic biomarker in human breast cancer. HER2 protein levels are higher in adenomas and carcinomas compared to normal mammary glands.	[76]
MUC1	MUC1 is a member of the mucin family present in both normal and tumor glandular epithelial cells. It has a polypeptide core with a side sugar chain and is abundant on the surface of breast cancer cells.	[77]
PR	The role of progesterone receptor (PR) protein continues to be studied in BC. The presence of PR protein in BC patients is associated with age. Loss of PR protein may contribute to BC.	[78]

BRCA1 has also been reported in familial BC, and homozygosity of *BRCA1* has been observed in some sporadic BC cases. Notably, normal tissues do not exhibit hypermethylation of the *BRCA1* gene [39].

A study has shown that *BRCA1* promoter hypermethylation is highly prevalent in BC and ovarian cancers, particularly in the Chinese population [40]. Hypermethylation of *BRCA1* is very commonly to found in BC and ovarian cancers [41]. For that, it was strongly suggested that *BRCA1* promoter hypermethylation has a significant role in BC prognosis and diagnosis [42].

Saliva is a source of cell-free DNA that can be isolated for various analyses, including DNA methylation examinations. Studies have utilized DNA from saliva to

investigate DNA methylation patterns in oral, head, and neck carcinoma [43]. In the context of BC DNA methylation tests have been conducted using both urine and saliva samples. Interestingly, significant alterations in the DNA methylation of the *BRCA1* gene were observed only in urine samples [44], while a study from 2013 reported changes in DNA methylation of BC-related genes isolated from saliva [45].

In 2002, a study conducted on German BC patients investigated the methylation status of several growth regulatory genes, including *CCND2*, *p16*, *RASSF1A*, and *14-3-3ó*. The findings revealed that promoter methylation is an early and recurrent event in BC, but it shows significant quantitative and gene-specific variations during tumour progression. Three biologically important genes, namely adenomatous polyposis coli (*APC*), *RASSF1A*, and death-associated protein (*DAP*) kinase gene, are typically unmethylated. The study was in 34 BC by high sensitivity methylation-specific polymerase chain reaction (PCR) and paired preoperative serum DNAs [46]. However, another study observed hypermethylation in these genes in a substantial percentage of BC samples. Specifically, *RASSF1A* exhibited hypermethylation in 65% of the samples, while *APC* and *DAP* kinase genes had hypermethylation in 47% and 50% of the samples, respectively [47]. These findings suggest that DNA hypermethylation of specific genes may be associated with various pathological features of BC.

The methylation status of BC-related genes, including *BRCA1*, retinoic acid receptor beta (*RARβ2*), *TWIST*, *CCN*, *p16*, and *E- cadherin* was studied in 193 BC patients to explore their association with clinical and pathological features. The study revealed varying degrees of methylation, with *CCND2* exhibiting methylation in 11% of the cases. This suggests that DNA methylation may be linked to different pathological characteristics of BC [48].

Studies have also shown that the methylation status of genes such as *APC* and *RASSF1A* in cell-free DNA isolated from the sera of BC patients can serve as a prognostic biomarker [49]. Furthermore, cell-free DNA from sera has been used to study DNA methylation of cancer-related genes in various cancer types [50-52].

In 2009, it was reported that DNA methylation of ADAM metallopeptidase domain 33 (*ADAM33*) in the promoter region could potentially serve as a useful molecular biomarker for distinguishing between invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC) [53].

The *E-cadherin* gene, located on chromosome 16, encodes a cell surface adhesion protein and plays a crucial role in maintaining cell-cell adhesion in epithelial tissues. Epigenetic silencing of the *E-cadherin* gene, characterized by CpG methylation, has been observed in some human BC cell lines [54]. Studies have shown that hypermethylation of the *E-cadherin* CpG islands is present in a significant percentage of DCIS and metastatic lesions, indicating a potential role of methylation in tumour progression [55].

Alterations in DNA Methylation and its Potential Clinical Utility in Patients with BC: A decade ago, scientists focused on DNA with abnormality in the serum of cancer patients was observed. Numerous studies and experiments revealed various types of changes in cell-free DNA obtained from BC patients' serum, blood, or plasma. Epigenetic changes, oncogene mutations, gene rearrangements, and aberrant promoter hypermethylation are examples of cell-free DNA modifications documented in serum and plasma [79]. Hypermethylated TSGs are frequently seen in BC tissues and can be diagnosed at an early stage of the illness therefore, alterations in DNA methylation have the potential to be used as both diagnostic and prognostic biomarkers [80].

DNA Methylation–Based Biomarkers in BC Patients:

Although DNA methylation analysis is an arising technique, there is a lack of a viable epigenetics test that uses bodily fluids “expect blood samples” for BC diagnosis and follow-up. Biomarkers based on DNA methylation can be found in saliva and urine. Although some success with urine and saliva, blood samples have shown the most promise of finding such biomarkers because they are easily accessible, therefore, DNA methylation biomarkers in blood are the most frequently used in both medical diagnosis and therapy. There is growing evidence that a panel of epigenetic biomarkers is required for increased sensitivity and specificity in BC recognition. Alteration in DNA methylation between healthy and malignant breast tissue may be used as both prognostic and diagnostic biomarkers in BC [81-83].

Analysis of DNA Methylation Extracted from Peripheral Blood: Several studies have investigated the potential clinical value of DNA methylation in peripheral blood as a biomarker for BC risk. The findings regarding gene-specific methylation and its association with BC risk are still uncertain. However, some studies have reported interesting results (Table 3).

One of these studies suggests that there are differences in the DNA methylation patterns of certain genes in patients with BC compared to healthy

Table 3: Several methylated genes in peripheral blood in female patients with BC.

Gene	Epigenetic alterations	Potential clinical utility	Ref.
VIM, CXCR4	Hypomethylation	Prognostic biomarker	[84]
DOK7	Hypermethylation	Prognostic biomarker	[84]
ATM	Hypermethylation	Risk biomarker	[85]
BRCA1	Hypermethylation	Risk biomarker	[86]
ESR1, TIMP3	Hypermethylation	Prognostic biomarker	[87]

Table 4: Several methylated genes in female and male patients from tissue biopsy with BC.

Gene	Epigenetic alterations	Potential clinical utility	Ref.
LYL1, SREBF1, ALX4, TP73, FEV, NEUROG1, TRIM29, HOXA11, PAX, SOX10, MGMT.	Hypermethylation	Prognostic biomarker	[88]
E-cadherin	Hypermethylation	Prognostic biomarker	[89]
CRY2	Hypomethylation	Prognostic biomarker	[90]
BRCA1, SCGB3A1, APC, CCND2, FOXA1, PSAT1, RASSF1A.	Hypermethylation	Prognostic biomarker	[91]
LAG-3, CTLA-4, PD-1, TIM-3	Hypomethylation	Prognostic biomarker	[92]
IL15RA	Hypermethylation	Prognostic biomarker	[93]
PRAC2, EFCAB1, WT1, BCL9, SMYD3, ANKRD53, HOXD9, ITIH5, ZNF154, ZNF177, TMEM132C, TDRD10, RNF220, RIMBP2.	Hypermethylation	Prognostic biomarker	[94]
KPNA2	Hypomethylation	Prognostic biomarker	[95]

individuals. Specifically, the vimentin (*VIM*) and C-X-C chemokine receptor type 4 (*CXCR4*) genes were found to be hypomethylated in patients with BC. *CXCR4* is a chemokine receptor that plays a role in cancer progression. On the other hand, the docking protein 7 (*DOK7)* gene was found to be hypermethylated in BC patients compared to controls. Based on these results, the study suggests that hypermethylation of the *DOK7* gene may have potential as a biomarker for diagnosing BC. In other words, detecting higher levels of DNA methylation in the *DOK7* gene could be indicative of the presence of BC. Additionally, the study suggests that the hypomethylation of the *VIM* and *CXCR4* genes may be used as biomarkers for predicting the prognosis of BC. Hypomethylation of these genes could potentially indicate a more favourable or unfavourable outcome in patients with BC [84]. Other studies have focused on evaluating promoter hypermethylation of TSGs, which are frequently methylated in BC. These studies suggested that methylation levels in *BRCA1* and *ATM* genes could serve as biomarkers for BC risk [85,86]. Furthermore, the methylation levels of estrogen receptor 1 gene (*ESR1*) and metallopeptidase inhibitor 1 (*TIMP3*) have been found to be higher in BC patients compared to controls, indicating their potential as biomarkers for BC risk [87]. However, it is important to note that only a limited number of studies have explored gene-specific DNA methylation as a risk biomarker for BC. Further research is necessary to determine the potential of DNA methylation as a tool for predicting BC risk.

Analysis of DNA Methylation in Tissue Biopsy: Several DNA methylated genes have been identified as potential prognostic biomarkers BC. Table 4 of the mentioned study summarizes the major findings related to the clinical value of DNA methylation in tissue biopsies. The study observed alterations in the methylation levels of various genes in BC tissues.

In BC tissues, the following genes were found to have altered methylation levels: lymphoblastomic leukemia 1 (*LYL1*), sterol regulatory element binding transcription factor 1 (*SREBF1*), ALX Homeobox 4 (*ALX4*), Tumor Protein P73 (*TP73*), FEV transcription factor (*FEV*), neurogenin 1 (*NEUROG1*), tripartite motif containing 29 (*TRIM29*), homobox A11(*HOXA11*), paired box 9 (*PAX9*), SRY-box transcription factor 10 (*SOX10*), and methylguanine-DNA-methyltransferase (*MGMT*). Promoter hypermethylation and reduced expression levels of these genes were observed in neoplastic tissues compared to healthy tissues, suggesting that DNA methylation alterations may be associated with tissue-specific susceptibility and BC progression [88]. Also, another study investigated the methylation profile of the *E-cadherin* gene promoter and found it to be hypermethylated in 94% of BC tissues, which was associated with an aggressive tumor phenotype in infiltrating BC [89]. Additionally, the study identified hypomethylation of the cryptochrome circadian regulator 2 (*CRY2*) gene in BC tissues, along with downregulated expression. This reduction in *CRY2* regulation was negatively associated with ER status, resulting in higher tumor grade and shorter survival time for BC patients [90]. The prognostic performance of DNA methylation promoters was evaluated for seven genes: *BRCA1,* secretoglobin family 3A member 1 (*SCGB3A1*), *APC*, *CCND2*, forkhead box A1 (*FOXA1*), phosphoserine

Table 5: Several methylated genes in liquid biopsies from female patients with BC.

Gene	Epigenetic alterations	Potential clinical utility	Ref.
SFN	Hypermethylation	Prognostic biomarker	[96]
DAPK	Hypermethylation	Risk biomarker	[97]
GSTP1, TIMB3	Hypermethylation	Prognostic biomarker	[98]
RASSF1A	Hypermethylation	Risk biomarker	[99]
CST6	Hypermethylation	Prognostic biomarker	[100]
BRCA1, FHIT	Hypermethylation	Risk biomarker	[101]
APC, RARβ	Hypermethylation	Risk biomarker	[102]
GSTP1	Hypermethylation	Prognostic biomarker	[103]

Table 6: Several methylated genes in TNBC.

Gene	Epigenetic alterations	Potential clinical utility	Ref.
BRCA1	Hypermethylation	Prognostic biomarker	[108]
WNT10A, MATK, ABLIM1, IFI35, FAM150B, CPT1A, SKOR1.	Hypomethylation	Prognostic biomarker	[109]
TSPAN9, ADAM12, VWCE	Hypomethylation	Prognostic biomarker	[110]

aminotransferase 1 (*PSAT1*), and *RASSF1A*. These genes were found to be hypermethylated compared to normal breast tissues, suggesting their potential as prognostic biomarkers in BC [91]. Furthermore, immune checkpoint genes, including lymphocyte Activating 3 (*LAG-3*), cytotoxic T-lymphocyte associated protein 4 (*CTLA-4*), programmed cell death 1 (*PD-1*), and hepatitis A virus cellular receptor 2 (*TIM-3*), showed hypomethylation of their promoters in BC patients compared to healthy breast tissues. This suggests that these modifications may serve as prognostic biomarkers in BC [92]. Hypermethylation of the IL15RA gene was reported in BC tissues compared to healthy breast tissues, indicating its potential as a biomarker [93]. Another study reported hypermethylation of *PRAC2,* calaxin (*EFCAB1*), wilms' tumour gene 1 (*WT1*), B-cell CLL/lymphoma 9 *(BCL9*), set and mYND domain containing 3 (*SMYD3*), ankyrin repeat domain 53 (*ANKRD53*), homeobox D9 (*HOXD9*), inter-alpha-trypsin inhibitor heavy chain 5 (*ITIH5*), zinc finger protein 154 (*ZNF154*), zinc finger protein 177 (*ZNF177*), transmembrane protein 132C (*TMEM132C*), tudor domain containing 10 (*TDRD10*), Ring finger protein 220 (*RNF220*), and RIMS binding protein 2 (*RIMBP2*) genes in BC patients compared to healthy tissues [94]. The methylation of the karyopherin subunit alpha 2 (*KPNA2*) gene was analyzed in male and female BC tissues, and promoter hypomethylation was associated with a lower survival rate, suggesting its potential as a prognostic biomarker [95]. It's important to note that these findings are specific to the study mentioned which is DNA methylation and may require further validation and research for clinical application.

Analysis of DNA Methylation in Liquid Biopsy: Several studies have investigated DNA methylation patterns using liquid biopsy in BC patients. Table 5 summarizes the results of DNA methylation analysis in various genes using liquid biopsy. The genes analyzed in these studies include stratifin (*SFN*), death-associated protein kinase 1 (*DAPK*), glutathione S-transferase pi 1 (*GSTP1*), TIMP metallopeptidase inhibitor 1 (*TIMP3*), *RASSF1A*, cystatin E/M (*CST6*), *BRCA1*, fragile histidine triad diadenosine triphosphatase (*FHIT*), *APC*, *RARβ*, and glutathione S-transferase pi 1 (*GSTP1*) [96-103]. The results consistently revealed hypermethylation in all these genes when analyzed in liquid biopsy samples. These findings indicate that DNA methylation alterations in these genes may have potential as biomarkers for assessing the risk or predicting the survival prognosis of breast cancer.

DNA Methylation Changes in TNBC: TNBC is the most aggressive subtype of BC compared to other subtypes and it is characterized by its clinicopathological features, including early onset, high risk of relapse, and a higher frequency of metastases to the lungs, liver, and central nervous system [104]. Epigenetic alterations, such as DNA methylation, are more common in TNBC compared to other subtypes of BC [105]. These alterations play a role in the development and progression of TNBC. Germinal *BRCA1* and *BRCA2* mutations are found in approximately 19.5% of TNBC cases, although this percentage may vary based on family history and ethnicity [106]. These mutations have implications for both prognosis and treatment options. Patients with metastatic TNBC have an unfavorable prognosis due to the absence of ER, PR, and HER2 protein expression. The absence of these receptors makes it challenging to develop targeted therapies, leading to higher mortality rates [107]. Table 6 summarizes the main findings related to the clinical value of DNA methylation in TNBC. The results of studies mentioned in [108-110] provide insights into the potential of DNA methylation as a prognostic and predictive biomarker in TNBC. It's important to note that TNBC is a complex and heterogeneous disease, and further research is needed to better understand the epigenetic alterations involved and their clinical implications. These findings suggest that DNA methylation analysis in TNBC may hold promise for improving risk assessment, prognosis prediction, and the development of targeted therapies.

DNA Methylation Changes May Induce Drug Resistance in BC Patients: DNA methylation plays a crucial role in

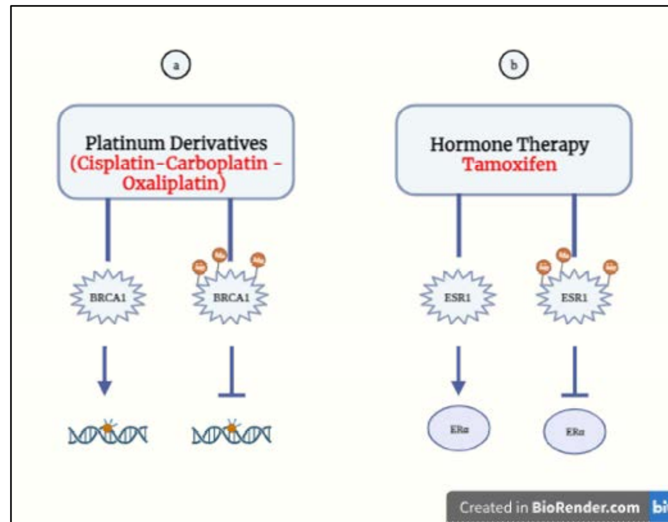

Fig. 3: Illustrates the effects of epigenetic alterations on treatments in breast cancer (BC) patients. (a) Platinum chemotherapy treatment aims to stimulate the *BRCA1* gene, which plays a crucial role in cellular damage repair. However, when there is hypermethylation in the promoter region of the gene, it leads to gene damage and inhibition of DNA repair processes. This can potentially reduce the effectiveness of platinum chemotherapy in BC treatment. (b) Tamoxifen, an anti-estrogen drug, functions by inhibiting the expression of estrogen receptor alpha (ERα). Methylation of the *ESR1* gene, which encodes ERα, determines the resistance to hormone therapy. When *ESR1* is methylated, it results in the absence of ERα expression, which is associated with reduced responsiveness to tamoxifen treatment.

clinical practice for patients with BC not only in early diagnosis but also in treatment stratification. The activation of the *BRCA1* gene is essential for cellular damage repair. However, when promoter hypermethylation occurs and gene activity is reduced, the ability of *BRCA1* to repair DNA cross-links is inhibited [111]. Patients with BC are being treated using chemotherapeutic agents, such as platinum and its derivatives (cisplatin, carboplatin and oxaliplatin), the capability of *BRCA1* to reform the DNA cross-links is inhibited (Fig. 3a). Therefore, *BRCA1* hypermethylation may turn into a predictive element for therapeutic treatments [112]. Methylation alterations can also disrupt the balance between estrogen receptor α (ERα) coactivators and core suppressors, leading to poor prognosis. Methylation of the *ESR1* gene, which encodes ERα, blocks the formation of ERα and can be used as a predictive biomarker for breast tumors that show a lack of response to hormone therapy (Table 3 & Fig. 3b) [113].

TNBC, which occurs in 10–20% of BC, refers to cancer cells that are negative for ER and PR hormones and HER2 protein and accounts for 15% to 20% of all invasive BC in Caucasian population (Table. 2) [114]. Cancer cells do not have receptors for estrogen, progesterone and HER2 protein (Fig. 4). The GeparSixto trial valued the effect of *MGMT* promoter methylation on the patients with TNBC treated with carboplatin therapy to monitor their response and survival time. 210 TNBC patients, divided into two groups (with and without carboplatin) no statistically significant difference in therapeutic response was noticed [115].

Tamoxifen, an anti-estrogen drug, is administered to BC patients to inhibit the dimerization and activation of ERα, thereby preventing relapse. Methylation of *ESR1* is associated with the loss of ERα expression and is closely linked to resistance to hormone therapy [116].

In HER2-positive BC patients, a study on human BC cell lines (SKBr3 and AU565) identified DNA methylation biomarkers associated with trastuzumab resistance. It was found that hypermethylation of the transforming growth factor β-induced (*TGFβI*) promoter leads to its silencing and resistance to trastuzumab [117]. Overall, DNA methylation alterations provide valuable insights for diagnosis, treatment stratification, and prediction of therapeutic response in BC patients.

In another study, the MDA MB 231 cell line was treated with doxorubicin, and it was found that *GSTP1* and *MGMT* exhibited hypomethylation, leading to an increase in gene expression levels. However, hypomethylation of *ESR1* in MDA MB 231 cells was

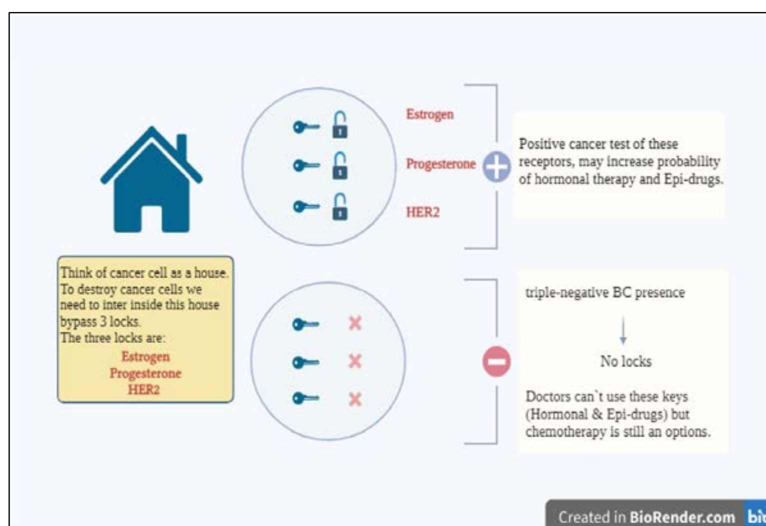

Fig. 4: Triple negative breast cancer TNBC. TNBC tumors do not express three receptors, making TNBC patients negative for estrogen, progesterone and HER2. These receptors status distinguishes TNBC from other types of BC and play a crucial role in the growth and development of BC cells and their absence in TNBC makes it more challenging to treat compared to other types of BC.

shown to halt the increase in gene expression. Additionally, when MDA MB 231 cells were treated with both doxorubicin and paclitaxel, a synergistic effect on matrix Metallopeptidase 9 (*MMP9*) gene expression was observed, which differed from the effects seen when each drug was administered alone. These findings suggest that the molecular alterations caused by doxorubicin or paclitaxel treatment do not always result in a synergistic effect on these genes, and further investigations are needed to determine their potential as prognostic and therapeutic response biomarkers [118].

Multiple studies have explored the combination of histone deacetylase inhibitors (HDACIs) and DNMTIs in BC. These studies have demonstrated a cooperative effect of combining these epi-drugs with anticancer therapies. In BC cell lines, the combination of DNMTIs and HDACIs has been shown to re-express ER [119, 120].

Recently, an increasing number of DNA methylation changes have been involved in gene regulation during breast tumor growth and metastasis. Epigenetic modulatory drugs, known as epi-drugs, primarily target HDACs and DNMTs [121]. In a study conducted in 2023, eribulin treatment was found to alter DNA methylation patterns and the expression of epigenetic modifiers, including ten-eleven translocation methylcytosine dioxygenase 1 (TET1), DNMT1, and DNMT3A/B, in TNBC cells. These results suggest that eribulin can modulate DNA methylation patterns in TNBC cells and may have therapeutic potential [122].

An experiment suggested that combining poly (ADP ribose) polymerase (PARP) inhibitors with DNMTIs enhances the cytotoxic effect of PARP in TNBC cell lines [123]. Additionally, it has been proposed that DNMTIs may induce homologous recombination deficiency in TNBC cells, like *BRCA*-mutant cancer cells, even in TNBC cells without *BRCAs* mutations [124].

In a preclinical study, BC cell lines were generated with tamoxifen resistance by decreasing the expression of *E-cadherin* through promoter hypermethylation. Treatment with the demethylating agent 5-azacytidine (AZA) resulted in *E-cadherin* demethylation and restored sensitivity to tamoxifen [125].

These findings highlight the potential of epigenetic alterations, such as DNA methylation modifications and the use of epi-drugs in BC research and therapeutic approaches. Further investigations are needed to fully understand the mechanisms and clinical implications of these alterations related with BC.

CONCLUSION

Several studies have demonstrated that epigenetic alterations, such as DNA methylation, play a role in BC [126-131]. These alterations can lead to changes in the expression of TSGs and oncogenes. Researchers have identified both hypomethylated and hypermethylated genes in the tissues and blood of both male and female BC patients. The detection of gene-specific methylation

through peripheral blood, tissue biopsy, and liquid biopsy has the potential to assist in early cancer diagnosis and monitoring the effectiveness of pharmacological treatments, enabling personalized and targeted therapy. Gene-specific DNA methylation can also influence the sensitivity of BC to chemotherapy, hormone therapy, and immunotherapy. Recent research has focused on epi-drugs, particularly for BC patients who are resistant to standard anticancer treatments. Combining DNMTIs and HDACIs with conventional therapies has shown promising results. Additionally, studying DNA methylation alterations can help identify prognostic biomarkers and improve therapeutic approaches for BC patients.

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