DNA methylation and breast cancer: A way forward (Review)

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Abstract. The current management of breast cancer (BC) lacks specific non-invasive biomarkers able to provide an early diagnosis of the disease. Epigenetic-sensitive signatures are influenced by environmental exposures and are mediated by direct molecular mechanisms, mainly guided by DNA methylation, which regulate the interplay between genetic and non-genetic risk factors during cancerogenesis. The inactivation of tumor suppressor genes due to promoter hypermethylation is an early event in carcinogenesis. Of note, targeted tumor suppressor genes are frequently hypermethylated in patient-derived BC tissues and peripheral blood biospecimens. In addition, epigenetic alterations in triple-negative BC, as the most aggressive subtype, have been identified. Thus, detecting both targeted and genome-wide DNA methylation changes through liquid-based assays appears to be a useful clinical strategy for early detection, more accurate risk stratification and a personalized prediction of therapeutic response in patients with BC. Of note, the DNA methylation profile may be mapped by isolating the circulating tumor DNA from the plasma as a more accessible biospecimen. Furthermore, the sensitivity to treatment with chemotherapy, hormones and immunotherapy may be altered by gene-specific DNA methylation, suggesting novel potential drug targets. Recently, the use of epigenetic drugs administered alone and/or with anticancer therapies has led to remarkable results, particularly in patients with BC resistant to anticancer treatment. The aim of the present review was to provide an update on DNA methylation changes that are potentially involved in BC development and their putative clinical utility in the fields of diagnosis, prognosis and therapy.

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Contents

1. Introduction

The accurate early diagnosis of breast cancer (BC) is critical for the management of the disease; however, there are currently no established non-invasive epigenetic biomarkers for BC diagnosis or screening. BC is the most frequent type of cancer (14%), after lung cancer (14.6%) (1), and has been reported to be the leading cause of cancer-associated death among females worldwide (2). BC accounts for ~40% of all types of cancer that develop after the age of 40 years, while 6.6% of cases arise prior to the age of 40 years (3). The prognosis of BC is associated with early diagnosis, which is detected using mammography (4), while the CA15-3 and CA27-29 markers are used for monitoring the recovery of the disease and the effectiveness of treatment (5,6).

Male BC has a low frequency and accounts for 1% of diagnoses annually (7). The incidence rate of male BC prior to the age of 50 years is 0.2 per 100,000 individuals, which increases to 6.3 per 100,000 individuals after the age of 65 years. This means that the risk of BC developing in males, as well as in females, increases with age (8).

Most cases of BC are considered sporadic in nature, as they are mainly associated with environmental factors (9); ~20% are familial, of which 5-10% are represented by cases of hereditary breast and ovarian cancer, due to mutations that are transmitted in an autosomal dominant manner. In total, ~25% of cases are due to germline mutations in major susceptibility genes, such as breast cancer (BRCA)1 and 2, while 1-3% of mutations occur in susceptibility genes, such as checkpoint

kinase 2, ATM serine/threonine kinase (ATM), phosphatase and tensin homolog, tumor protein p53 (TP53) and partner and localizer of BRCA2 (10,11).

BC is highly heterogeneous at both the histological and molecular levels. In fact, in the beginning, it was studied as a complex multi-step process based on genetic alterations with the subsequent activation of cellular proto-oncogenes and/or inactivation of tumor suppressor genes. The interplay between genetic and environmental risk factors is guided by precise epigenetic programs, mainly by DNA methylation changes, leading to a dysregulation of key molecular pathways involved in breast carcinogenesis (12). Differences in DNA methylation profiles between patients with BC and healthy controls may aid in clarifying the molecular basis of BC development and, potentially, provide useful prognostic and/or diagnostic biomarkers (13). For instance, global hypomethylation and hypermethylation of CpG islands are molecular events that occur early in patients with BC, suggesting potential regions of sensitivity to disease onset (14). In addition, most DNA methylation changes were reported during the progression from healthy breast tissue to ductal carcinoma *in situ* (DCIS), while the epigenetic changes from DCIS to invasive BC were minimal. Therefore, DNA methylation changes are involved in early carcinogenesis and may represent a marker for early diagnosis (15).

Recently, it has been observed that DNA methylation profiles may be mapped, not only in tumor-specific tissue and peripheral blood biospecimens, but also by extracting circulating tumor (ct)DNA from plasma using liquid-based assays, allowing the evaluation of both primary lesion and metastases (16). Detection of ctDNA may be used both as an early screening for cancer and for the diagnosis of minimal residual disease. Furthermore, it may be useful to monitor the response to pharmacological treatments, for a personalized therapeutic strategy. This method is based on the possibility of isolating ctDNA from the blood, thus overcoming the challenges associated with tissue biopsy, due to tumor localization and/or the small size of the sample recovered. ctDNA detection is easy to perform and represents a source of valuable information on the biology of tumors and a minimally invasive test used to evaluate epigenetic alterations (2).

The aim of the present review was to update the pathogenic DNA methylation signatures emerging from studies performed on tissue samples, peripheral blood and ctDNA using liquid-based assays, from both female and male patients with BC, including triple-negative BC (TNBC). The potential role of these signatures as novel prognostic and diagnostic biomarkers, as well as alternative drug targets in patients with therapy resistance, is also discussed.

2. Focus on basic mechanisms of DNA methylation

DNA methylation changes are widely influenced by environmental events, such as aging, lack of physical activity, stress, depression, high alcohol consumption and air pollution, as well as biological processes, such as inactivation of the X chromosome, genomic imprinting, reprogramming of the genome during differentiation, development and survival, and genetic/molecular alterations (17,18). All of these events, individually or in combination, are sensitive to the development of neoplasms and influence its course (19). The DNA methylation process consists of the addition of a methyl group to the pyrimidine ring of cytosine in the CpG dinucleotides, which is mediated by DNA methyltransferase (DNMT) enzymes. In general, methylated DNA mediates transcriptional repression of tissue-specific genes. By contrast, hypomethylation favors an increase in gene expression, leading to the activation of specific genes. When the promoter region is methylated, gene expression is reduced, as the proteins within the chromodomain are able to bind to the methylated groups and prevent the recruitment of activator proteins. The promoters and CpG islands, corresponding to actively expressed genes, are generally hypomethylated in disease as compared with those in healthy subjects (20); the same may occur in patients with cancer, in whom there may be a focal hypermethylation of the promoter due to the presence of CpG islands in the first exonic region of almost half of the genes that cause genomic instability. Several tumors develop due to methylation of numerous CpG islands within the genome or a secondary event to somatic genetic mutations in regulatory genes (21). DNA methylation is regulated in a tissue-specific manner, but it is also largely influenced by the genomic context (22). As a general paradigm, DNA hypermethylation at regulatory regions, such as promoters and CpG islands, may repress the transcription of genes, thereby acting as a tumor suppressor. However, a positive association between DNA methylation levels, at both the intragenic (introns and exons) and intergenic regions (enhancers), and gene expression features is gaining relevance due to its impact on cancer risk. On the other hand, the different effects of DNA methylation in the promoter vs. the gene remain to be further clarified (23).

3. DNA methylation changes and their potential clinical utility in patients with BC

Certain tumor suppressor genes are frequently hypermethylated in BC tissues and are detected in the early disease stage (24). DNA methylation changes between healthy and malignant breast tissue may be considered as both prognostic and diagnostic biomarkers in BC.

Analysis of tissue biopsy

Gene-specific DNA methylation changes as prognostic biomarkers. Several gene-specific DNA methylation changes have been identified, suggesting that epigenetic alterations may have prognostic value in BC. In Table I, major results regarding the potential clinical value of DNA methylation in tissue biopsy are reported.

Among these, there is E-cadherin, which is involved in cell-cell adhesion via its association with catenins. The silencing of the E-cadherin gene by genetic or epigenetic changes leads to tumorigenesis. The methylation profile of the E-cadherin gene promoter was mapped in 50 BC tissues as compared with that in 50 normal breast samples. In agreement with previous studies, the results indicated hypermethylation of the E-cadherin promoter in 94% of tissues, with an association with an aggressive tumor phenotype in infiltrating BC (25).

Avraham *et al* (26) performed a study on specific DNA methylation between healthy breast tissue and normal tissues, and in tumor breast tissue and other neoplastic tissues, such as colon, lung and endometrial cancer. The methylation profile

Table I. Gene-specific methylation in tissue from female and male[a] patients with breast cancer.

Author (year)	Epigenetic alteration	Gene	Potential clinical utility	Ref.
Shargh *et al* (2014)	Hypermethylation	E-cadherin	Prognostic biomarker	(25)
Avraham *et al* (2014)	Hypermethylation	ALX4, FEV, HOXA11, LYL1, NEUROG1, PAX, MGMT, SOX10, SREBF1, TP73, TRIM29	Prognostic biomarkers	(26)
de Almeida *et al* (2019)	Hypermethylation	WT1, BCL9, SMYD3, ZNF154, ZNF177, HOXD9, ITIH5, TMEM132C, TDRD10, RNF220, RIMBP2, PRAC2, EFCAB1, ANKRD53	Prognostic biomarkers	(27)
Salta *et al* (2018)	Hypermethylation	APC, BRCA1, FOXA1, PSAT1, CCND2, RASSF1A, SCGB3A1	Prognostic biomarkers	(2)
Yang *et al* (2019)	Hypermethylation	IL15RA	Prognostic biomarker	(28)
Mao *et al* (2015)	Hypomethylation	CRY2	Prognostic biomarker	(29)
Sasidharan Nair *et al* (2018)	Hypomethylation	PD-1, CTLA-4, TIM-3, LAG-3	Prognostic biomarkers	(30)
Cui *et al* (2020)	Hypomethylation	KPNA2	Prognostic biomarker	(31)

[a]In all studies, the subjects were female, apart from Cui *et al* (31), where subjects were female/male.

of certain genes was observed to be altered in neoplastic breast tissue, including the ALX homeobox 4 (ALX4), FEV transcription factor, ETS family member (FEV), homeobox A11 (HOXA11), LYL1 basic helix-loop-helix family member (LYL1), neurogenin 1 (NEUROG1), paired box 9 (PAX9), O-6-methylguanine-DNA methyltransferase (MGMT), SRY-box transcription factor 10 (SOX10), sterol regulatory element binding transcription factor 1 (SREBF1), tumor protein P73 (TP73) and tripartite motif 29 (TRIM29) genes. Specifically, in healthy tissues, ALX4, FEV, HOXA11, LYL1, NEUROG1, PAX9, MGMT, SOX10, SREBF1 and TP73 exhibited promoter hypomethylation, while in neoplastic tissues, including the mammary gland, promoter hypermethylation and reduced expression levels were present. In addition, TRIM29 promoter hypomethylation in normal breast tissue and hypermethylation in other healthy tissues was observed. In the neoplastic breast tissue, there was hypermethylation of the promoter with reduced gene expression, while hypomethylation was observed in the other neoplastic tissues. This suggested that epigenetic alterations may be associated with tissue-specific susceptibility and may be involved in cancer progression (26).

De Almeida *et al* (27) analyzed DNA methylation and gene expression profiles in BC tissue and matched normal tissue. In addition to WT1 transcription factor (WT1), zinc finger protein (ZNF)154, BCL9 transcription coactivator, homeobox D9 (HOXD9), SET and MYND domain containing 3 (SMYD3), inter-α-trypsin inhibitor heavy chain 5 (ITIH5) and ZNF177, methylation was found in seven other genes, namely ring finger protein (RNF), transmembrane protein 132C (TMEM132C), tudor domain containing (TDRD10), EF-hand calcium binding domain 1 (EFCAB1), RIMS binding protein 2 (RIMBP2), ankyrin repeat domain 53 (ANKRD53) and PRAC2 small nuclear protein (PRAC2), also known as C17orf93, of which no association with cancer has been previously observed. These authors reported hypermethylation in breast tissue as compared with that in healthy tissue. Furthermore, the RNF220, TMEM132C, TDRD10, EFCAB1, RIMBP2, ANKRD53 and PRAC2 genes had promoter hypermethylation with subsequent reduction in gene expression as compared with that in healthy tissue. To evaluate the prognostic ability of the CpG sites, survival curves were generated. Of the seven genes, only PRAC2, TDR10 and TMEM132C had significant prognostic value. In fact, they exhibited a different regulation of gene expression between tumor and healthy breast tissue, as well as an association with poor prognosis. In particular, the gene expression level of PRAC2 was upregulated in tumor tissue, while there was a decrease in TMEM132C and TDR10, despite promoter hypermethylation (27).

Furthermore, the prognostic performance of promoter-related DNA methylation was evaluated in seven genes, including adenomatous polyposis coli (APC), BRCA1, forkhead box A1 (FOXA1), phosphoserine aminotransferase 1 (PSAT1), cyclin D2 (CCND2), ras association domain family member 1 (RASSF1A) and secretoglobin family 3A member 1 (SCGB3A1), which were hypermethylated in 137 breast tissues as compared to normal breast tissue. Disease-specific survival curves and disease-free survival curves were drawn for methylation status, which revealed that higher DNA methylation levels were associated with shorter disease-specific survival (2).

Another study suggested that IL-15 receptor α (IL15RA) hypermethylation may be associated with the development and progression of BC by regulating the expression levels of other genes. By comparing the methylation data of 316 breast tumor tissues with 21 healthy breast tissues, it was observed that

hypermethylation of IL15RA led to upregulation of the proline rich 11 (PRR11), nucleolar and spindle-associated protein 1 (NUSAP1) and homeobox C11 (HOXC11) genes and a reduced regulation of the SH3 and cysteine rich domain 2 (STAC2) genes. Using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, it was determined that PRR11, NUSAP1, STAC2 and HOXC11 methylation was associated with the 'cell adhesion-related molecular pathway' and, therefore, BC progression (28).

The involvement of DNA methylation in regulating the cryptochrome circadian regulator 2 (CRY2) gene, which has a key role in breast tumorigenesis, was observed, suggesting a strong association with BC progression. The methylation of the CRY2 gene was evaluated in 1,881 patients with BC and was observed to be hypomethylated, with the downregulation of gene expression in BC tissues, as compared with that in healthy tissue. This reduction in CRY2 regulation was due to estrogen receptor (ER) negativity, resulting in a higher tumor grade and shorter survival time for patients with BC (29).

The expression levels of different immune checkpoint genes, including T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), programmed cell death protein-1 (PD-1), lymphocyte activating 3 (LAG-3) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), were analyzed in 8 breast tumor tissues and 8 healthy breast tissues. Upregulation was associated with hypomethylation of the promoter in breast tumor tissues as compared with that in healthy breast tissues, suggesting that these modifications may be utilized as prognostic biomarkers in BC (30).

Methylation of the karyopherin α-2 (KPNA2) gene was also analyzed in 33 male and female BC tissues, as compared with that in 20 healthy tissues. The KPNA2 protein is a nuclear import factor that is involved in nucleocytoplasmic transport, cell proliferation, migration and invasion in tumors. The association between KPNA2 expression and prognosis in BC was evaluated using overall survival, relapse-free survival, distant metastasis-free survival and post-progression survival time curves in patients with BC. KPNA2 may serve as a potential indicator of poor prognosis, as promoter hypomethylation of the gene was reported in patients with a lower survival rate (31,32).

The WT1 gene and promoter methylation was analyzed in a panel of normal breast epithelial and BC tissues. Contrary to previous reports, WT1 was indicated to be hypermethylated and expressed in 32% of BC tissue, but not in normal breast epithelium, suggesting that WT1 may not have a tumor suppressor role in BC (33).

In both male and female BC, promoter hypermethylation in tumor tissue was frequently determined to be high in the BRCA2, mutS homolog 6, WT1, PAX5 and 6, cadherin 13, GATA binding protein 5, killin, P53 regulated DNA replication inhibitor, thrombospondin 1, glutathione S-transferase pi 1 (GSTP1), MGMT, TP53, TP73, estrogen receptor 1 (ESR1), CD44 antigen (CD44), cell adhesion molecule 1, retinoic acid receptor β (RARB), PYD and CARD domain containing, Von Hippel-Lindau tumor suppressor (VHL), cyclin dependent kinase inhibitor 2A (CDKN2A), ATM, checkpoint with forkhead and ring finger domains (CHFR), RB transcriptional corepressor 1 (RB1), BRCA1 and serine/threonine kinase 11 (STK11) genes. Compared with that in females, promoter methylation of the VHL, ESR1, CDKN2A, CD44, CHFR, BRCA2, RB1 and STK11 genes was lower in males. This appears to be due to the presence of higher levels of estrogen in females compared with those in males. This suggests that there are differences in male and female breast carcinogenesis with respect to promoter methylation (34).

Analysis of DNA extracted from peripheral blood

Global DNA methylation changes as risk biomarkers. Several studies (35-41) have evaluated global DNA methylation levels in blood using various methods and BC samples and controls, and concluded that a high level of methylation was associated with poor survival time, while lower methylation was associated with improved prognosis (35). The strategies included measuring the percentage of methylated DNA using a luminometric methylation assay (LUMA), measuring the methylation of repetitive DNA elements (LINE-1, Alu or Sat2) using pyrosequencing, the concentration of 5-methyldeoxycytosine (5-mdC) using liquid chromatography-mass spectrometry and the MethyLight assay, as surrogates of global DNA methylation levels.

Global hypomethylation has been suggested to cause genomic instability and lead to an increase in cancer risk. A total of three studies using the LUMA assay were performed between 2012 and 2014 (36-38), which reported different results. Specifically, Delgado-Cruzata *et al* (36) determined similar global DNA methylation levels between BC cases and controls, while Xu *et al* (37) observed low global DNA methylation levels in BC cases, and Kuchiba *et al* (38) observed high global DNA methylation in patients with BC. In addition, Delgado-Cruzata *et al* (36) measured the methylation of repetitive DNA elements and detected low levels in patients with BC compared with those in the controls.

Other studies, performed using different methods, have evaluated the methylation level of LINE-1 repeats and the results provided a consensus opinion, namely that there were no differences between BC cases and healthy controls. Cho *et al* (39) observed global hypomethylation in patients with BC using the 5-mdC assay. This diversity of response depends not only on the method used, but also on the timing with which the sampling was performed and the time taken to perform the analysis. In fact, various factors may influence the results. For instance, if the sampling was performed while the subjects were undergoing therapeutic treatment, this may easily alter the result. In addition, the presence of the disease may lead to a different distribution of the cells in the bloodstream (39). Other studies have been performed comparing global DNA methylation levels and the risk of cancer, with inconsistent results (40,41); certain studies concluded that there was no significant association between global DNA methylation levels and BC risk, while others revealed a positive association between methylation level and BC risk. This suggests that the association between global DNA methylation and BC risk remains to be further clarified (40).

In a previous study, the association between global DNA methylation and physical activity or global DNA methylation and BC risk was evaluated. A higher level of global methylation in patients performing physical activity over longer periods of time was observed. In addition, lower cancer risk was determined in patients with a higher level of global methylation. Furthermore, where possible, global DNA methylation levels were evaluated from a peripheral blood sample 3 years

Table II. Gene-specific methylation in peripheral blood from female patients with breast cancer.

Author (year)	Epigenetic alteration	Gene	Potential clinical utility	Ref.
Cho *et al* (2015)	Hypermethylation	BRCA1	Risk biomarker	(39)
Brennan *et al* (2012)	Hypermethylation	ATM	Risk biomarker	(42)
Widschwendter *et al* (2008)	Hypermethylation	HYAL2, NUP155, ZNF217, PTGS, TITF1, NEUROD1 SFRP1	Risk Biomarkers	(43)
Shirkavand *et al* (2018)	Hypermethylation Hypomethylation	DOK7 VIM, CXCR4	Prognostic biomarkers	(44)
Zmetakova *et al* (2013)	Hypermethylation	ESR1, TIMP3	Prognostic biomarkers	(45)

All cases were female.

prior to the onset of cancer. It was observed that higher levels of methylation reduced the risk of developing the neoplasm. Therefore, this study suggested that long-term physical activity was associated with global DNA methylation and that the latter was associated with a decreased risk of BC (41).

Gene-specific DNA methylation as risk biomarkers. Various studies have evaluated the methylation levels of tumor suppressor genes associated with BC development by analyzing leukocyte DNA from patients with BC and in controls. The evaluation of DNA methylation in leukocytes is of particular importance, as peripheral blood is easier to obtain at multiple time-points than tissues. Furthermore, it allows the identification of early risk markers, even in healthy individuals, and allows the easy evaluation of differences in methylation levels between healthy individuals and patients with disease. In Table II, major results regarding the potential clinical value of DNA methylation in peripheral blood are reported.

The role of gene-specific methylation in peripheral blood, as a marker of BC risk, is uncertain. Certain studies have evaluated whether promoter hypermethylation of tumor suppressor genes, which are frequently methylated in BC, may be used as a biomarker for BC risk. BRCA1 and ATM promoter methylation was analyzed in 1,021 BC samples and 1,036 controls, and in 640 BC samples and 741 controls, respectively. Hypermethylation was observed in patients with BC, indicating that DNA methylation levels in BRCA1 and ATM may serve as a marker of BC risk and, as peripheral blood is a comparatively more accessible biospecimen, this may be valuable in epigenome-wide association studies (39,42). In a case-control study including 1,083 patients with BC, it was investigated whether DNA methylation was associated with BC risk. The risk of cancer onset was indicated to be increased when the methylation levels of the hyaluronidase 2 (HYAL2), nucleoporin 155 (NUP155), ZNF217, post-transcriptional gene silencing (PTGS), thyroid transcription factor-1 (TITF1), neuronal differentiation 1 (NEUROD1) and secreted frizzled related protein 1 (SFRP1) genes were low (43). To date, only a small number of studies have evaluated gene-specific DNA methylation as risk biomarkers. Further studies are required to determine whether DNA methylation may be a new tool to predict the risk of BC.

Gene-specific DNA methylation changes as prognostic biomarkers. Shirkavand *et al* (44) investigated the DNA methylation status of the docking protein 7 (DOK7), VIM, C-X-C chemokine receptor type 4 (CXCR4) and SAM pointed domain-containing ETS transcription factor genes in 60 patients with BC and 40 controls. The percentage of promoter methylation changes in the patients with BC and normal specimens were analyzed using a gel analyzer software (GenAnalyzer 2010). The results suggested that the VIM and CXCR4 genes, the latter of which is a chemokine receptor involved in cancer progression, were hypomethylated in patients with BC as compared with that in the healthy individuals. Hypermethylation of the DOK7 gene in BC vs. controls was present. It was indicated that hypermethylation of the DOK7 gene in patients with BC may be used as a biomarker for cancer diagnosis, and that VIM and CXCR4 hypomethylation may be used as a biomarker for BC prognosis (44). To determine whether the DNA methylation level in peripheral blood may be used as a prognostic biomarker, DNA methylation levels in the ESR1 and TIMP metallopeptidase inhibitor 1 (TIMP3) genes were analyzed in patients with BC and controls. Different results have been obtained: Zmetakova *et al* (45) determined promoter hypermethylation in patients with BC compared with that in the controls, while Widschwendter *et al* (43) did not observe any significant changes in the methylation levels between the controls and patients with BC.

Analysis of ctDNA and circulating tumor cells (CTCs) using liquid biopsy

Epigenetic analysis may be performed on circulating material, such as CTCs and ctDNA using liquid biopsy, starting from a simple and non-invasive blood sample. It is an advantageous procedure, easy to perform with high specificity and sensitivity in minimal residual disease quantification, and is able to monitor the response to therapy and/or any resistance (46). CTCs are rare cells with variable morphology and may be obtained from primary BC or from free metastasis in the bloodstream, which invade other tissues or organs and cause injury. It is also possible for CTC groups called 'clusters' to be present, resulting in more aggressive metastases (47). Numerous studies have analyzed genes with specific methylation using liquid biopsy. In Table III, major results about the potential clinical value of DNA methylation in liquid biopsy were reported. DNA methylation patterns may be used as a biomarker for the risk and survival prognosis of patients with cancer.

Gene-specific DNA methylation changes as risk biomarkers. The development of cancer has been indicated to be associated with epigenetic changes. A previous study estimated the ctDNA methylation levels of fragile histidine triad diadenosine triphosphatase (FHIT) and BRCA1 promoters in 30 patients with BC and 30 healthy individuals. Statistical analysis was used to analyze the differences in the methylation levels between the groups and the results suggested that the methylation levels of the BRCA1 and FHIT promoters were higher in the patients with BC compared with those in the healthy individuals. The FHIT methylation level was also indicated to be associated with BC and may be useful for BC diagnosis (48).

Death-associated protein kinase 1 (DAPK) and RASSF1A methylation was evaluated in 26 patients with BC, 16 patients with benign breast disease and 12 age-matched healthy controls. APC and RARB methylation was also analyzed in 121 patients with BC, 79 patients with benign breast disease and 66 healthy volunteers. Statistical analysis was performed to analyze the positivity rates of the investigated genes. DAPK, RASSF1A, APC and RARB genes had higher hypermethylation frequencies in patients with BC compared with those in the controls (49-51), suggesting that they may be valuable biomarkers for BC detection. Further studies confirming these results and the markers may be useful in a clinical setting in the diagnosis and management of BC.

Gene-specific DNA methylation changes as prognostic biomarkers. In a recent study on CTCs, promoter hypermethylation of certain tumor suppressor genes, including RASSF1A, CCND2, GSTP1, HIC ZBTB transcriptional repressor 1 (HIC1), RARβ and DAPK, was analyzed. In particular, the methylation of the RASSF1A gene was associated with BC progression and metastases, while GSTP1 methylation was associated with chemotherapy treatment response and patient survival time (52). Chimonidou *et al* (53) evaluated whether the DNA methylation status in CTCs and ctDNA was comparable and whether it reflected the status of the primary tumor. The study compared the methylation status in both CTCs and ctDNA in three genes, SOX17, cystatin E/M (CST6) and BRMS1 transcriptional repressor and anoikis regulator (BRMS1) in 153 patients with BC and healthy individuals. CST6, a tumor suppressor gene, has been associated with the inhibition of proliferation, migration, invasion and bone metastases in BC. SOX17 has been associated with the regulation of the Wnt/β-catenin signaling pathway. BRMS1 is involved in chromatin remodeling. A correlation between CTCs and ctDNA for the SOX17 promoter methylation was both observed in patients with early and metastatic BC, but not for CST6 and BRMS1. DNA methylation analysis of SOX17 may thus be of prognostic value (53).

In a study using different methylated specific CTC clusters, hypomethylation in binding sites in the octamer-binding transcription factor 4 (OCT4), nanog homeobox (NANOG), SOX2 and SIN3 transcription regulator family member A (SIN3A) genes, which have been associated with transcription, proliferation and stability, was observed and associated with poor prognosis. In the same study, in an experiment on single cells, application of reagents that led to cluster dissociation caused a change in DNA methylation with hypermethylation in the binding sites of OCT4, SOX2, NANOG and SIN3A and the consequent reduction of gene expression. This may be due to increased intracellular calcium and the loss of cell-to-cell junction after treatment of CTC clusters with dissociating compounds (54).

For certain genes, the degree of methylation in the CTC regions matched that observed in the neoplastic tissue, BC cell lines and peripheral blood, suggesting a more aggressive neoplasm. In particular, promoter hypermethylation of the RASSF1A, GSTP1 and APC genes was reported in tissue and BC cell lines, in the CCND2 and RARβ genes in tissue, and in the HIC1, DAPK1 and TIMP3 genes in BC cell lines. Furthermore, BRCA1 and ESR1 promoter hypermethylation in tissue, BC cell lines and peripheral blood was also observed (2,32,35,34,39,45).

Other studies using ctDNA have confirmed an association between high methylation levels in certain tumor suppressor genes and poor prognosis in BC. It was reported that the stratifin (SFN), GSTP1, CST6 and TIMP3 genes were always hypermethylated in BC samples (55-57).

4. DNA methylation changes in TNBC

An estimated 10-20% of BC cases are classified as TNBC. This is the most aggressive type of BC compared with the other subtypes, due to its clinicopathological characteristics, including early onset, relapse and higher frequency of developing lung, liver and central nervous system metastases. Patients with metastatic TNBC have unfavorable prognosis, as their cells do not express the ER, progesterone receptor and HER2. The absence of these receptors still makes it difficult to formulate a targeted therapy with a consequently higher mortality rate (58). Germinal BRCA1 and BRCA2 mutations occur in 19.5% of TBNC, which may vary according to family history and ethnicity (59). Epigenetic alterations in TNBC are more frequent than in other subtypes of BC (60).

Gene-specific DNA methylation changes as prognostic biomarkers. A study including 119 BC samples and 118 healthy samples analyzed the methylation profiles of genes whose methylation pattern identifies the TNBC subtype. Out of these, seven were indicated to be differentially methylated and associated with clinical conditions. Overall survival analysis of the selected methylated genes in BC was performed and the family with sequence similarity 150, member B, maturase K, interferon-induced protein 35, Wnt family member 10A and SKI family transcriptional corepressor 1 genes were determined to be hypomethylated, with gene upregulation, and were associated with favorable patient survival. In addition, actin binding LIM protein 1 and carnitine palmitoyltransferase 1A had low gene expression and were associated with poor patient outcome. According to a KEGG analysis, the 'cell movement', 'cell proliferation' and 'cell differentiation processes' pathways were associated with these genes, supporting the roles of these seven differentially methylated genes as potential markers for TNBC prognosis (61).

A recent study also analyzed several hypomethylated genes in 50 patients with TNBC and 24 healthy controls. In the TNBC tissues, promoter hypomethylation in ADAM metallopeptidase domain 12 (ADAM12), tetraspanin 9 (TSPAN9) and Von Willebrand factor C and EGF domains (VWCE) genes was observed. The VWCE gene promoted cancer development

Table III. Gene-specific methylation in liquid biopsies from female patients with breast cancer.

Author (year)	Epigenetic alterations	Gene	Potential clinical utility	Ref.
Liu *et al* (2015)	Hypermethylation	BRCA1, FHIT	Risk biomarker	(48)
Ahmed *et al* (2010)	Hypermethylation	DAPK	Risk biomarker	(49)
Kloten *et al* (2013)	Hypermethylation	RASSF1A	Risk biomarker	(50)
Swellam *et al* (2015)	Hypermethylation	APC, RARB	Risk biomarker	(51)
Bao-Caamano *et al* (2020)	Hypermethylation	RASSF1A, GSTP1	Prognostic biomarkers	(52)
Chimonidou *et al* (2017)	Hypermethylation	CST6, SOX17, BRMS1	Prognostic biomarkers	(53)
Zurita *et al* (2010)	Hypermethylation	SFN	Prognostic biomarker	(55)
Radpour *et al* (2011)	Hypermethylation	GSTP1, TIMB3	Prognostic biomarkers	(56)
Chimonidou *et al* (2013)	Hypermethylation	CST6	Prognostic biomarker	(57)

All cases were female.

Table IV. Gene-specific methylation in triple-negative breast cancer.

Author (year)	Epigenetic alteration	Genes	Potential clinical utility	Ref.
Chen *et al* (2019)	Hypomethylation	MATK, IFI35, FAM150B, SKOR1, WNT10A, ABLIM1, CPT1A	Prognostic biomarkers	(61)
Mendaza *et al* (2020)	Hypomethylation	ADAM12, TSPAN9, VWCE	Prognostic biomarkers	(62)
Zhu *et al* (2015)	Hypermethylation	BRCA1	Prognostic biomarker	(63)

All cases were female.

and progression. The TSPAN9 gene was associated with cell development, tumor proliferation and invasion, while ADAM12 was associated with proteolytic process, apoptosis, cell cycle and cell adhesion. ADAM12 hypomethylation was associated with a more unfavorable prognosis than the other two genes. Furthermore, ADAM12 knockdown decreased TNBC cell proliferation and migration, suggesting that it may be a potential therapeutic target (62).

The role of BRCA1 gene methylation in 239 TNBC cases was analyzed to investigate the association between clinical data and BRCA1 gene methylation. Of note, BRCA1 DNA methylation was observed in 57.3% of cases. Multivariate analyses further indicated that BRCA1 promoter methylation was an independent predictor of overall survival and disease-free survival. In addition, the BRCA1 promoter was also associated with a significant decrease in overall survival time, suggesting BRCA1 promoter methylation may serve as a biomarker for TNBC prognosis (63).

Furthermore, it was demonstrated that Tet methylcytosine dioxygenase 1 (TET1) was specifically overexpressed in patients with TNBC and associated with a shorter overall survival time. This revealed a previously uncharacterized role of TET1 as an oncogene, with hypomethylation and the activation of oncogenic signaling pathways. Several previous studies in BC have reported TET1 to be a tumor suppressor; thus, there is evidence suggesting that TET1 may function as both an oncogene and a tumor suppressor, depending on the cellular context (64).

Another study reported that the expression of the ganglioside GD3 (GD3) gene was markedly higher in patients with ER-negative BC compared with that in patients with ER-positive BC and also highly expressed in TNBC compared to other types of BC. This increase in expression was associated with the hypomethylation of the ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 1 gene. Elevated expression of GD3 in human BC cells increased their proliferation, migration, invasion and colony formation ability, suggesting that GD3 may be a potential prognostic biomarker in TNBC (65). In Table IV, the main results regarding the potential clinical value of DNA methylation in TNBC are presented.

5. DNA methylation changes may induce drug resistance in patients with BC

Gene-specific DNA methylation in patients with BC represents a valuable tool in clinical practice, which contributes not only to the early diagnosis of the disease, but also to risk stratification and therapeutic treatment (66). It is well-known that activation of the BRCA1 gene leads to cellular damage repair, which is highly compromised when epigenetic alterations occur with subsequent promoter hypermethylation and reduction in gene activity (Fig. 1A). In the treatment of patients with BC using chemotherapeutic agents, such as platinum and its derivatives (cisplatin, carboplatin and oxaliplatin), the ability of BRCA1 to repair the DNA cross-links is inhibited. Therefore, BRCA1 hypermethylation may become a predictive

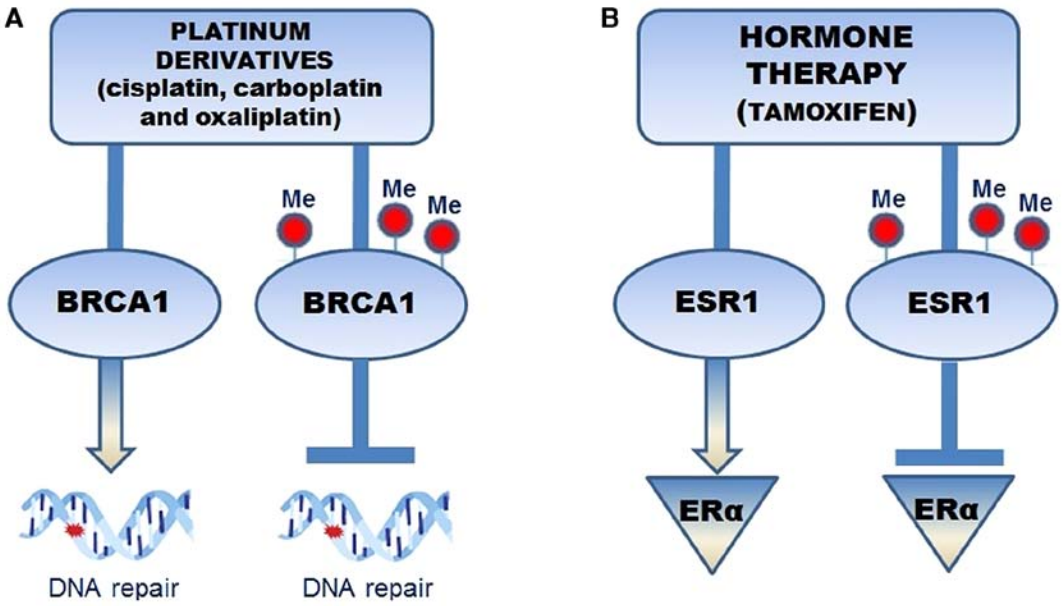

Figure 1. Effects of epigenetic alterations on treatments in patients with BR. (A) Chemotherapy treatment with platinum derivatives induces the BRCA1 gene to trigger cellular damage repair. Epigenetic alterations, with promoter gene hypermethylation, cause impairment of gene activity with inhibition of DNA repair capacity. (B) Tamoxifen inhibits ERα expression, preventing the onset of relapse. The methylation of ERS1 determines the absence of ERα expression with subsequent therapy resistance. BC, breast cancer; BRCA1, BC gene 1; ESR1, estrogen receptor 1; ERα, estrogen receptor α; Me, methyl group.

element for therapeutic treatments (67). Epigenetic alterations may disrupt the balance between ERα coactivators and corepressors and have been associated with poor prognosis and endocrine therapy resistance (68).

ESR1 methylation, which codes for ER-α, appears to represent a predictive biomarker for the endocrine treatment efficacy in breast tumors that do not respond to hormone therapy. It has been observed that tamoxifen, an anti-estrogen drug, administered to patients with BC, inhibited ER-α dimerization and activation, preventing relapse. ERS1 methylation determined the absence of ER expression and was associated with hormone therapy resistance (69) (Fig. 1B). Furthermore, one study performed on human BC cell lines (SKBr3 and AU565) evaluated the epigenetic biomarkers associated with trastuzumab resistance in patients with HER2-positive BC. It was indicated that hypermethylation of the transforming growth factor β-induced (TGFBI) promoter led to epigenetic silencing of the gene and trastuzumab resistance (70).

The GeparSixto trial evaluated the effect of MGMT promoter methylation on the response and survival time of patients with TNBC treated with carboplatin therapy. A total of 210 TNBC tumors were divided into two therapy groups, namely those with and without carboplatin. No statistically significant difference in therapeutic response was observed (71). DNA methylation changes among two BC cell lines, MCF-7 and MDA-MB-231 (TNBC), were evaluated after administration of doxorubicin and paclitaxel. In the treated MCF-7 cells, promoter methylation changes were observed, with changes in cyclin A1 and prostaglandin-endoperoxide synthase 2 gene expression. In the MDA-MB-231 cells, ESR1 hypomethylation was observed to not increase gene expression. In cells treated with doxorubicin only, GSTP1 and MGMT hypomethylation was observed with an increase in gene expression levels. Furthermore, in MDA-MB-231 cells treated with doxorubicin and paclitaxel, a synergistic effect on MMP9 gene expression was observed, which was different from that in the cells treated with doxorubicin or paclitaxel alone. The molecular changes observed suggested that doxorubicin or paclitaxel administration does not always produce a synergistic effect and further studies are required to consider them as prognostic and therapeutic response markers (72).

6. Epi-drugs in combination therapy for BC treatment

Recently, attention has focused on the epi-drugs used to overcome epigenetic alterations and hormonal therapy resistance, such as DNMT inhibitors (DNMTIs). The DNMTIs decitabine (DAC) and 5-azacytidine (AZA) are agents involved in DNA demethylation (73).

Several studies combining the efficacy of DNMTIs, histone deacetylases inhibitors (HDACIs) and vorinostat, have been performed in BC. A synergistic effect of both epigenetic drugs with anticancer therapy and of the combination of the epi-drugs themselves was determined (74,75). Initially, DAC and AZA had a synergistic effect in combination with HDACIs in preclinical and clinical studies in a different cancer type (76); however, no synergistic effect between AZA and entinostat was observed in a phase II clinical trial in patients with hormone-refractory BC (77). The combination of HDACIs and DNMTIs in BC cell lines resulted in re-expression of ER. In a preclinical study, BC cell lines with resistance to tamoxifen were generated, leading to promoter hypermethylation of E-cadherin with decreased expression. After AZA administration, E-cadherin demethylation was observed, along with re-established sensitivity to tamoxifen (78).

A further experiment suggested that administration of DNMTIs with poly (ADP-ribose) polymerase (PARP)-inhibitors enhanced the cytotoxic effect of PARP inhibition in TNBC cell lines (79). In addition, it was suggested that DNMTIs may cause homologous recombination deficiency in BRCA wild-type TNBC cells, similar to BRCA-mutant cancer cells (80).

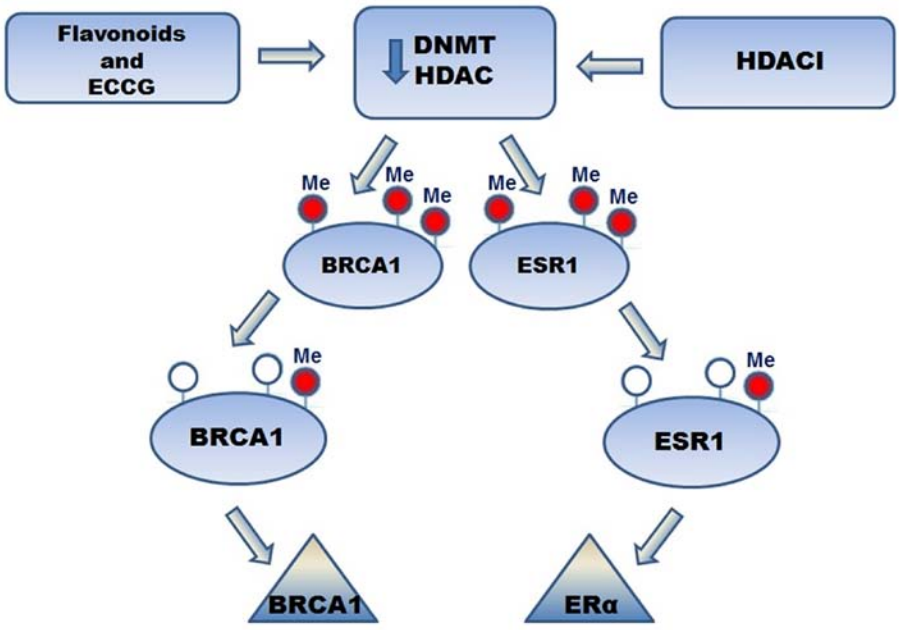

Figure 2. Flavonoids, EGCG and HDACI inhibit the activity of DNMT and HDAC. This leads to significantly decreased BRCA1 and ESR1 promoter methylation with induction of BRCA1 and ERα expression. EGCG, epigallocatechin-3-gallate; HDACI, HDAC inhibitors; DNMT, DNA methyltransferase; HDAC, histone deacetylases; BRCA1, breast cancer gene 1; ESR1, estrogen receptor 1; ERα, estrogen receptor α; Me, methyl group.

Several studies have demonstrated that isoflavone intake leads to neoplasm reduction in BC. Epigallocatechin-3-gallate (EGCG) is one of most studied flavonoids in BC. It acts as an inhibitor of DNMT and HDAC. It also induced the expression of tumor suppressor genes, leading to a reduction in the development of metastasis and cancer progression. Treatment with EGCG and HDACIs led to re-expression of ERα in ER-negative BC. Furthermore, EGCG induced ER re-expression in ER-negative BC and has decreased DNMT activity in BC cell lines. Regarding the effect of EGCG and flavonoids on BRCA1 and ESR-1 promoter methylation, a significant decrease in BRCA1 and ESR-1 promoter methylation was observed, which led to increased expression levels in ER-negative BC (81) (Fig. 2). In another study, the possibility of converting aggressive TNBC cells into a less aggressive phenotype using epigenetic drugs was analyzed (82). Guadecitabine (DNMTI) and entinostat (HDACI) had antitumor effects in patient-derived xenograft mouse models (82).

Despite these encouraging results, to date, the use of epi-drugs has marked limitations due to adverse reactions, including a high degree of cytotoxicity (13,83). This has not stopped their use; however, further investigation is required to determine the correct dose between epi-drugs and/or anticancer treatments and to open new possibilities to increase the number of targets and personalized therapies, with a considerable reduction of side effects.

7. Conclusion

Numerous studies have reported epigenetic alterations, such as DNA methylation, which led to changes in expression of oncogenes and tumor suppressor genes in patients with BC (84). Studies performed on tissues and whole blood have detected several hypo- and hypermethylated genes in both male and female patients with BC.

Detection of gene-specific methylation using liquid biopsy may facilitate early cancer diagnosis and assist with monitoring pharmacological treatment in order to obtain a personalized and targeted therapy. The sensitivity to treatment using chemotherapy, hormone and immunotherapy may be altered by gene-specific DNA methylation. In recent studies, attention has focused on epigenetic drugs. In particular, the association of the demethylation agents DNMTI and HDACI, administered alone and/or in combination with anticancer therapies, has led to remarkable results, particularly in patients with BC and resistant to anticancer treatment. Furthermore, studies on epigenetic alterations represent a valid tool for the search for prognostic biomarkers and for improving therapeutic treatments for patients with cancer.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

MTV and CN conceived the study. GDE, GB and GC performed the literature search/selection drafted the manuscript and prepared the figures. MTV, GF, GFN and CN revised and edited the manuscript. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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