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Review

Aberrant promoter CpG methylation and its translational applications in breast cancer

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Abstract

Breast cancer is a complex disease driven by multiple factors including both genetic and epigenetic alterations. Recent studies revealed that abnormal gene expression induced by epigenetic changes, including aberrant promoter methylation and histone modification, plays a critical role in human breast carcinogenesis. Silencing of tumor suppressor genes (TSGs) by promoter CpG methylation facilitates cells growth and survival advantages and further results in tumor initiation and progression, thus directly contributing to breast tumorigenesis. Usually, aberrant promoter methylation of TSGs, which can be reversed by pharmacological reagents, occurs at the early stage of tumorigenesis and therefore may serve as a potential tumor marker for early diagnosis and therapeutic targeting of breast cancer. In this review, we summarize the epigenetic changes of multiple TSGs involved in breast pathogenesis and their potential clinical applications as tumor markers for early detection and treatment of breast cancer.

Key words Breast cancer, tumor suppressor gene, CpG, methylation, tumor marker

Breast cancer is the most prevalent tumor and a major cause of morbidity and mortality among women worldwide[1]. Over the past decades, there has been a significant increase in breast cancer incidence, with more than one million new cases each year[2]. Diagnosis of breast cancer at the early stage results in a high survival rate (~98%), whereas diagnosis at the advanced stage results in a significantly lower survival rate (~27%)[3]. Due to the lack of early detection methods for breast cancer, current therapies are necessary but not sufficient to improve the survival of women with breast cancer. Thus, identification of tumor markers for the early detection and therapeutic targeting of breast cancer is essential.

Although much progress has been made in understanding the biology of breast cancer, its etiology is still not very clear. Activation of oncogenes and inactivation of tumor suppressor genes (TSGs) synergistically contribute to the cancer progression. Early studies have demonstrated that genetic alterations, such as chromosomal translocations and point mutations, are responsible for TSG inactivation. Recently, accumulating evidence indicates that epigenetic alterations provide an alternative yet important mechanism for TSG silencing. Epigenetic modifications include DNA methylation and histone modifications, which cooperatively affect chromatin structure and genomic stability[4,5]. Epigenetic modifications play important roles in the regulation of cell cycle, apoptosis, signal transduction, and tumorigenesis[6].

A series of TSGs silenced by promoter CpG methylation have been identified in breast cancer, indicating aberrant methylation of TSGs as a key factor in breast cancer pathogenesis. In addition, breast cancer usually progresses gradually from a less aggressive, hormone-dependent to a highly invasive, hormone-

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independent phenotype [7,8], implying that silencing of hormone-mediated TSGs occurs in breast cancer progression. Frequent TSG methylation in breast cancer makes it a potentially useful marker for disease diagnosis. Here, we summarize some recent studies on epigenetic alterations in breast cancer and further discuss their biological and clinical implications.

Aberrant Promoter Methylation Silences Critical TSGs in Breast Carcinoma

Although inactivating mutations of TSGs have been well documented in familial breast cancer, the incidence of mutations in sporadic breast cancer is rare. Yet, a large body of evidence has demonstrated that epigenetic aberration is a key player in silencing a variety of TSGs, which triggers breast tumor progression [9]. Identification of an epigenetic gene profile for breast cancer will thus be helpful to elucidate the molecular mechanisms underlying breast cancer pathogenesis.

As shown in Table 1, a series of promoter-methylated key TSGs involved in breast tumorigenesis have been reported, including genes for cell cycle regulation (*p16INK4a*, *p14ARF*, *p15*, *14-3-3σ*, *cyclin D2*, *p57KIP2*) , DNA repair [glutathione S-transferase pi 1 (*GSTP1*), O-6-methylguanine-DNA methyltransferase (*MGMT*), breast cancer 1 (*BRCA1*), human mutL homolog 1 (*hMLH1*)], hormone and receptor-mediated cell signaling pathways [estrogen receptor alpha (*ERα*), progesterone receptor (*PR*), retinoic acid receptor beta (*RARβ*), Ras association domain family member 1 (*RASSF1A*), spleen tyrosine kinase (*SYK*), TGFβ receptor II (*TGFβ RII*), high in normal 1 (*HIN1*), normal epithelial cell-specific 1 (*NES1*), suppressor of cytokine signaling 1 (*SOCS1*), secreted frizzled-related protein 1 (*SFRP1*), WNT inhibitory factor 1 (*WIF1*)], apoptosis [adenomatous polyposis coli (*APC*), death-associated protein kinase (*DAPK1*), hypermethylated in cancer 1 (*HIC1*), homeobox A5 (*HOXA5*), TWIST homolog of drosophila (*TWIST*), target of methylation-induced silencing (*TMS1*)], cell adhesion and metastasis [cadherin 1 (*CDH1*), cadherin 13 (*CDH13*), APC, tissue inhibitor of metalloproteinases 3 (*TIMP3*)], angiogenesis [maspin, thrombospondin 1 (*THBS1*)], and other processes. These epigenetic changes may lead to chromosomal instability, accumulated mutations in critical cell signaling pathways, ultimately contributing to breast cancer progression. Our group has also identified a series of TSGs silenced or down-regulated by promoter CpG methylation in breast cancer, such as *WIF1*, phospholipase C delta 1 (*PLCD1*), CKLF-like MARVEL transmembrane domain containing 5 (*CMTM5*), CKLF-like MARVEL transmembrane domain containing 3 (*CMTM3*), opioid binding protein/cell adhesion molecule-like (*OPCML*), ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), deleted in liver cancer 1 (*DLC1*), interferon regulatory factor 8 (*IRF8*), and dapper homolog 1 (*DACT1*), that are involved in breast tumor cell cycle control, apoptosis, and metastasis[10-16].

Clinical Implications of Promoter Methylation in Breast Cancer

Understanding the critical roles of promoter CpG methylation–mediated transcriptional repression/silencing in breast cancer led us to consider its potential clinical applications. Here, we further discuss the usage of DNA methylation markers for early cancer detection and prognosis prediction, as well as the application of demethylation drugs in breast cancer therapy.

Aberrant promoter methylation as a marker for early detection and prognosis in breast cancer

Women diagnosed with early-stage breast cancer have a better prognosis and require less severe treatment regimens than those diagnosed with advanced stage diseases. With the development of magnetic resonance imaging (MRI) and digital mammography, the accuracy of breast cancer detection has distinctly increased. However, current screening methods lack sensitivity and specificity[17], and new methods with higher sensitivity, specificity, and lower invasiveness are urgently required. Using tumor markers thus provides a valuable alternative approach.

In recent years, many studies demonstrated that aberrant DNA methylation has strong potential to serve as a novel tumor marker for early diagnosis and progression evaluation. First, aberrant promoter methylation of a series of TSGs is a common feature of malignancy; compared to gene mutations and copy number alternations, it occurs more frequently as an early event in tumorigenesis. Importantly, different types of tumors present distinctive promoter methylation profiles, which can improve specificity and sensitivity for tumor detection. Second, aberrant promoter methylation patterns can be detected even when they are embedded in an excess amount of normal DNA molecules. Third, techniques required for the detection of methylation patterns, such as methylation-specific PCR (MSR), MethyLight, and quantitative multiplex methylation-specific PCR (QM-MSP), are relatively simple, rapid, non-radioactive, and sensitive.

A number of cancer-specific genes have been found to be frequently methylated in breast cancer. These epigenetic biomarkers show promise for distinguishing between malignant and benign disease or normal tissue[18-23]. Further, considering that no single methylated gene was detected in any breast cancer types, it is

Table 1. Summary of major tumor suppressor genes methylated in breast cancer

Gene name	Chr location	Reported percentage of methylation in breast tumors	Reported percentage of methylation in breast cancer cell lines	Major functions	Reference(s)
ATM	11q22–q23	78%	–	DNA repair, cell cycle regulator	[60]
ABCB1	7q21.12	–	–	Multidrug resistance	[61,62]
APC	5q21–q22	36%–44%	44%	Cell polarity and chromosome segregation	[63,64]
BECN1	17q21	–	–	Autophage	[65]
BIN1	2q14	18%	100%	Apoptosis	[66]
BMP6	6p24–p23	–	–	Regulate TGFβ signaling pathway	[3]
BRCA1	17q12–21	51%	–	Cell cycle regulator, DNA repair, transcription regulation, apoptosis	[67,68]
CMTM5	14q11.2	–	100%	Apoptosis	[14]
CMTM3	16q22.1	18%	44%	Apoptosis	[10]
C/EBPδ	8p11.2–p11.1	–	–	Cellular proliferation, differentiation, metabolism, inflammatory response	[69]
CDKN1C	11p15.5	–	85%	Cell cycle regulator	[70]
CST6	11q13	48%–60%	–	Inhibit cystein proteases activity	[71,72]
CDH3	16q22.1	–	–	Calcium-dependent adhesion	[62,73]
CDH13	16q24.2–3	33%	35%	Cell adhesion, proliferation, metastasis	[74]
COX2	1q25.2–q25.3	35%	–	Cyclooxygenase-2; inflammation, mitogenesis	[75]
CDO1	5q23.2	–	–	Unknown	[76]
CIDEA	18p11.2	53%	86%	Caspase-independent cell death	[77]
Cyclin D2	12p13	46%	–	Cell cycle regulator	[78]
DBC1	9q32–33	26	33%	Unknown	[77]
DAPK	9q34.1	–	–	Apoptosis	[79]
DKK1	10q11.2	19%	27%	Wnt pathway inhibitor	[80]
DKK3	11p15.2	61.30%	71%	Wnt pathway inhibitor	[81]
DLC1	8p22	36%	22%–33%	Signal transduction, cell adhesion	[15,82]
ER	6q25.1	49%	–	Estrogen receptor	[83–85]
E-cadherin	16q22.1	48%	38%–55%	Cell adhesion, proliferation, metastasis	[75,86]
EMILIN2	18p11.3	44%	56%	Extracellular matrix glycoproteins	[77]
EDN3	20q13.2–q13.3	70%	83%	Unknown	[87]
EPHA5	4q13.1	64%	–	Unknown	[88]
FLJ25161	3p14.1	57%	75%	Unknown	[89]
FBXW7	4q31.3	50%	–	Cell cycle control	[90]
FBLN2	3p25.1	34%	56%	Cell motility and invasion	[77]
FOXC1	6p25	50%	–	Embryonic development	[62]
GADD45a	1p31.2–p31.1	67%	67%	Growth arrest and DNA repair	[91]
GSTP1	11q13	31%	44%	Glutathione transferase activity	[92]
HER4	2q34	–	–	Tyrosine kinase-type cell surface receptor	[93]
HIC1	17p13.3	–	–	Transcription Factor	[94]
HIN1	5q35–qter	65%	–	Cell communication,signal transduction	[95,96]
HMLH1	3p21.3	14%	–	DNA repair	[62]
HOXD11	2q31.1	75%	100%	Transcription factor, morphogenesis	[89]
HOXA5	7p15–p14	80%	–	Transcription regulation	[97]
IGF2	11p15.5	–	–	Unknown	[98]
IRF8	16q24.1	36%	66%	Transcription factor	[16]
KV1.3	1p13.3	42.30%	–	Apoptosis	[99]
OPCML	11q25	91%	90%	Cell adhesion	[11]
PCDH10	4q28.3	43%	88%	Apoptosis, metastasis, invasion	[89]
P3H2	3q28	42%	46%	A family of collagen prolyl hydroxylases required for proper collagen biosynthesis, folding, and assembly	[100]

(*To be continued*)

Table 1. Summary of major tumor suppressor genes methylated in breast cancer (*continued*)

Gene name	Chr location	Reported percentage of methylation in breast tumors	Reported percentage of methylation in breast cancer cell lines	Major functions	Reference(s)
P3H3	12q13	26%	31%	A family of collagen prolyl hydroxylases required for proper collagen biosynthesis, folding, and assembly	[100]
PLCD1	3p22–p21.3	52%	78%	Phospholipase activity, cell cycle arrest, metastasis, invasion	[13]
PCDHGB6	5q31	62%	75%	Cell adhesion	[89]
PGR	11q22	–	–	Progesterone receptor	[101]
PPP2R2B	5q32	56%–65%	–	Cell growth and division control	[102]
p16/CDKN2A	9p21	0–67%	33%	Cell cycle arrest	[103–108]
PTEN	10q23.3	22%–76%	–	Cell cycle arrest, apoptosis, cell adhesion, migration	[62,109]
RARB	3p24	–	–	Tumor suppressive activity	[110,111]
RRDM2	1p36.21	–	–	Apoptosis, metastasis	[112]
RASSF1A	3p21.3	62%	100%	Cell cycle control, apoptosis, DNA repair	[113–115]
SIM1	6q16.3–q21	75%	100%	Unknown	[89]
SALL1	16q12.1	63%	67%	Unknown	[77]
SFRP1	8p11–12	40%–75%	30%–100%	Wnt antagonist	[80,116,117]
SFRP2	4q31.3	77%	100%	Wnt antagonist	[80]
SFRP5	10q24.1	71%–73%	90%–91%	Wnt antagonist	[80,118]
SOX17	8q11.23	80.6%	–	Growth inhibition, cell cycle arrest	[119]
SRBC	1p15	60%	–	Unknown	[120]
SYK	9q22	32%	30%	Growth inhibition, metastasis	[121]
TIMP3	22q12.3	27%	29%	Metastasis, invasion	[122]
TWIST	7p21.2	5.6%–32%	–	Transcription regulator, EMT	[95,123]
TMS1	16p11.2–12.1	41%	77%	Apoptosis	[124]
TGM2	20q12	44.4%	5.7%–73.1%	Cell migration	[125]
TRIP10	19p13.3	–	–	Cell migration	[126]
THRB	3p24.2	100%	40%	Thyroid hormone receptor	[127]
WIF1	12q14.3	67%	80%	Wnt signaling inhibitor	[12]
XT3	3p21.3	43%	75%	Unknown	[89]
14-3-3δ	2p25.1	83%–96%	50%	Cell cycle arrest, metastasis	[128,129]

–, not available.

necessary to use cancer-specific methylation marker panels to screen breast cancer[24]. For example, a three-methylated-gene panel (*Cyclin D2*, *RARβ* , and *TWIST*) was successfully used to detect malignant breast cancer cells in ductal fluids[25]. A four-methylated-gene panel (*RASSF1A*, *TWIST*, *HIN1*, and *Cyclin D2*) with a high level of sensitivity and specificity was used to detect malignant breast tissues[26] as well. Compared to conventional ductal lavage cytology, a nine-methylated-gene panel (*RASSF1A*, *TWIST*, *HIN1*, *Cyclin D2*, *RARβ* , *APC*, *BRCA1*, *BRCA2*, and *p16*) was developed to double the sensitivity for breast cancer detection[27].

Cancer-specific methylation can also be extended to assess risk and predict prognosis for breast cancer. For example, frequently methylated *RASSF1A* and *APC*, as well as methylated *GSTP1* and *SFRP1*[28,29], were associated with poor outcome[28,29]. Recently, subtype-specific methylation markers were used to evaluate optional targeted treatments in breast cancer[30]. Using an array-based methylation assay, Holm *et al*.[31] uncovered a subtype- specific methylation pattern for distinguishing different breast tumor phenotypes, which will provide more detailed information for predicting breast cancer prognosis.

Additionally, blood-based detection of cancer-specific methylated DNA in breast cancer has shown potential for early detection and prognostic prediction[32-35].

Radpour *et al*.[3] found significant promoter methylation of seven genes [*APC*, bridging integrator 1 (*BIN1*), bone morphogenetic protein 6 (*BMP6*), *BRCA1*, cystatin 6 (*CST6*), *P16*, and *TIMP3*] in serum and tumor tissues from patients with breast cancer by using

MALDI-TOF mass spectroscopy.

Together, these studies show that cancer specific methylation changes in tumor tissues, plasma, and other fluids can be used as tumor markers for risk assessment and early diagnosis of breast cancer. More effective and simplified approaches for detecting methylated genes are being developed to increase the sensitivity and specificity of early detection and prognosis of breast cancer[36-38].

Aberrant promoter methylation as therapeutic target for breast cancer

Unlike genetic changes in cancers, gene silencing due to DNA methylation changes can be reversed by pharmacological demethylation. Thus, reactivating epigenetically silenced cancer genes and restoring their tumor suppression functions provide new insight for cancer therapy. Moreover, targeting epigenetic alterations also provides alternative ways for breast cancer preventative care, novel anticancer therapeutics, and drug investigation.

DNA methyltransferase (DNMTs) inhibitors, 5-aza-cytidine (5-Aza-CR) and 5-aza-2'-deoxycytidine (5-Aza-dC), are the first discovered epigenetic drugs. These compounds act by incorporating into DNA in place of the natural base, cytosine, during DNA replication, leading to covalent trapping of DNMTs[39]. This causes the depletion of active DNMTs and demethylation of genomic DNA. These reagents have already been approved by the US Food and Drug Administration (FDA) for treatment of a myelodysplastic syndrome (MDS), malignant mesothelioma, preleukemic disease, breast cancer, nasopharyngeal carcinoma (NPC) and other diseases[40-43]. Recently, Zebularine, a novel DNMT inhibitor with low toxicity and high selectivity for tumor cells, was reported to reactivate key genes silenced in breast cancer cell lines even at low doses[44]. In addition, other demethylation approaches, including DNMT inhibition via siRNA, ribozymes, and antisense oligonucleotides, have also been proposed but are still in their infancy. Some of these agents have been demonstrated to be promisingly effective in cell culture systems, animal models, and even clinical trials, whereas having little effect on normal cells [45]. These agents include MG98, an antisense oligonucleotide to DNA methyltransferase 1 [46,47], and RG108 [48,49], a novel small molecule that binds to the catalytic site of DNA methyltransferases.

The combination of histone deacetylases inhibitors (HDACIs), such as TSA and phenylbutyrate [50], with DNMT inhibitors is particularly valuable for cancer treatment. Furthermore, loss of estrogen receptor (ER) expression due to aberrant DNA methylation and histone modifications results in resistance to anti-estrogen therapy in breast cancer. Studies showed that combination of 5-azacytidine with TSA could induce re-expression of functional ER, thereby sensitizing ERα-negative breast cancer cells to tamoxifen therapy [51,52]. Combined with HDACIs, trastuzumab (Herceptin®), a humanized monoclonal anti-HER2 antibody, produced a synergistic effect on cell growth repression and apoptosis induction in breast cancer cells[53,54].

Furthermore, combinations of epigenetic drug treatments with conventional chemotherapeutic reagents or natural dietary ingredients could potentially work synergistically to increase therapeutic effects. A preclinical study has shown that 5-Aza-dC in combination with docetaxel, an anti-mitotic chemotherapeutic regent, could produce synergistic anti-cancer effects on breast cancer lines[55]. 5-Aza-dC combined with either amsacrine or idarubicin also showed promising efficacy. The green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCG), can cause the chromatin structural remodelling of ERα promoter by altering histone acetylation and methylation status, thereby resulting in ERα reactivation. Combined with TSA, EGCG reactivated multiple methylation-silenced TSGs by directly and indirectly inhibiting the enzymatic activities of DNMTs [56]. Dietary sulforaphane (SFN) [57], a histone deacetylase inhibitor, significantly inhibited the viability and proliferation of breast cancer cells but not normal cells *in vitro*.

Therefore, combined demethylation therapies may offer better therapeutic strategies for breast cancer. For example, clinical trials of trastuzumab combined with HDACI are in progress for locally advanced breast cancer [58], and a phase II breast cancer trial combining valproic acid (VPA) with FEC100 (5-fluorouracil, epirubicin, and cyclophosphamide) is ongoing[59].

Conclusions

In summary, substantial evidence demonstrates that epigenetic alterations, especially promoter CpG methylation of TSGs, play critical roles in breast tumorigenesis. Significant advances have been made for early detection biomarkers, risk assessment, prognostic prediction, and drug development in breast cancer. With the development of new epigenomic techniques and further investigations, a better perspective of the epigenetic profile of breast cancer will soon be revealed.

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