CHAPTER

9

Modulation of the Epigenome (Methylome) to Improve Chemotherapeutic Efficacy

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Abstract

Current clinical management of cancer typically includes cytotoxic chemotherapy—in the neo-adjuvant setting prior to surgery, in the adjuvant setting following surgery, or as a first-line treatment in patients that present with advanced disease. However, cancer cells often display de novo resistance to chemotherapeutic drugs or develop resistance with treatment. Once a cancer becomes chemoresistant, progression and demise of the patient become inevitable. New strategies that combine epigenetic therapies and cytotoxic chemotherapy show promise for more effective treatments which may lead to improvements in long-term patient outcomes. Demethylating drugs can sensitize cancer cells to chemotherapy, resulting in enhanced cell killing. New studies have shown that this effect is related to re-expression of genes encoding proteins of pro-apoptotic pathways, rendering cancer cells susceptible to cell killing by chemotherapeutic drugs. With further investigation of epigenetic mechanisms of chemoresistance and sensitization in cancer cells, new clinical strategies for management of cancer patients will emerge.

ABBREVIATIONS

5-aza	5-Aza-2′-deoxycytidine
BRCA1	Breast cancer 1
DNMT3b	DNA methyltransferase 3 beta
ERa	Estrogen receptor alpha
HDAC	Histone deacetylase
miRs	MicroRNAs
PR	Progesterone receptor
RARb2	Retinoic acid receptor beta 2
RNAi	RNA interference
TIMP-3	Tissue inhibitor of metalloproteinases 3
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor

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9.1 INTRODUCTION

Chemotherapy has long been a mainstay of systemic cancer treatment [1]. Even today, many cancer patients present with advanced disease that has spread from the primary site of occurrence into the vasculature, the lymphatics, or to distant tissue sites [2]. Once cancer becomes a systemic disease (metastatic cancer), surgical interventions are ineffective and/or incapable of cure. In fact, patients that present with early-stage (localized) invasive disease often have micrometastases that become evident following surgical removal of the primary cancer. Hence, cytotoxic chemotherapy has utility for treatment of patients with advanced disease (where the whole body is treated), as well as patients with early-stage disease to eliminate micrometastatic spread (adjuvant chemotherapy) (Fig. 9.1). In current cancer management, patients with localized disease may be treated with neo-adjuvant chemotherapy to shrink the primary cancer prior to surgery, resulting in more effective removal of the neoplastic lesion (Fig. 9.1).

Management of patients with chemotherapy is complex as there are numerous morbidities associated with treatments using cytotoxic drugs. The effectiveness of chemotherapeutic drugs tends to be directly associated with the dose of drug utilized. Hence, patients are typically treated with the maximum tolerated dose. In addition, patients typically receive combination chemotherapy which targets multiple pathways in the cancer cells. Durable responses to chemotherapy result in subsets of patients, although the underlying mechanism for chemosensitivity is not always evident. In some cases, the apparent success of adjuvant chemotherapy may actually reflect the effectiveness of surgical removal of the primary cancer (Fig. 9.1). Despite the use of high-dose combination chemotherapy regimens, many patients fail therapy. Some cancers display a natural resistance to specific classes of chemotherapeutic agents, rendering them largely ineffective at cell killing, and others evolve to develop resistance, resulting in cancer cell clones that cannot be killed by the chemotherapeutic drug combination. The development of chemoresistance is associated with progression of neoplastic disease and represents a harbinger of the demise of the patient as treatment fails and the cancer spreads.

Numerous mechanisms have been implicated in the development of chemoresistance (Fig. 9.2). In general, resistant cancer cells avoid contact with the drug by decreasing drug uptake and/or increasing drug efflux. With less intracellular drug, DNA damage (or interaction with other drug targets) that might lead to cell death is minimized. Cancer cells can also become resistant to chemotherapy by increasing DNA repair mechanisms to address the DNA damage that results from drug exposure with genotoxic agents (Fig. 9.2). With effective DNA repair, cancer cells avoid death despite drug exposure. Furthermore, alterations in mechanisms of apoptosis can result in chemoresistance. Increased expression of anti-apoptotic proteins (and pathways) protects cancer cells from drug-induced programmed cell death. Likewise, decreased expression of pro-apoptotic proteins (and pathways) protects cancer cells from programmed cell death (Fig. 9.2).

Several genetic mechanisms leading to chemoresistance in cancer cells have been elucidated. In this chapter, epigenetic mechanisms of chemoresistance and the use of epigenetic drugs to sensitize cancer cells to chemotherapy will be discussed. This discussion will focus on breast cancer as a cancer model system, DNA hypermethylation as a prominent epigenetic mechanism, and demethylating drugs that demonstrate potential strategies for overcoming chemoresistance. In particular, this chapter will present evidence that epigenetic

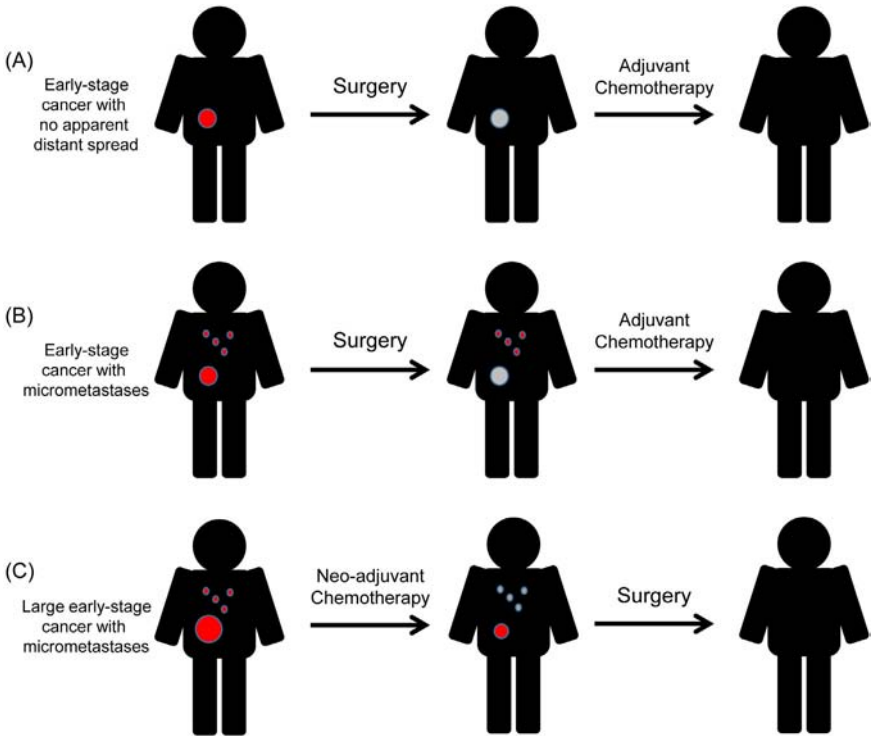

FIG. 9.1 **Utilization of cancer chemotherapy strategies and ideal/potential patient outcomes.** (A) In patients with an early-stage cancer that is small and is not associated with distant spread (micrometastases or other), surgery can often provide a cure. However, adjuvant chemotherapy is typically employed to treat any cancer that might be present, but is clinically undetectable. (B) Many patients present with early-stage cancer that is associated with microscopic spread (micrometastases). These micrometastases are not realized until after surgery when they expand in size. Adjuvant chemotherapy can eliminate these micrometastases in some cases, resulting in cure when combined with surgical removal of the primary cancer. (C) Patients that present with a large primary cancer (with or without micrometastases) may be treated with neoadjuvant therapy to shrink the primary cancer prior to surgery. Neoadjuvant therapy might also eliminate micrometastases, resulting in cure once surgery is performed.

loss of pro-apoptotic proteins and pathways accounts for drug resistance in breast cancer cell lines that display an aberrant hypermethylation phenotype and that treatment with demethylating drugs results in re-expression of the associated apoptotic genes concurrent with increased chemosensitivity.

9.2 DNA METHYLATION IN CANCER

DNA methylation (chemical alteration of cytosine bases) is the most studied and best understood mechanism of epigenetic regulation [3,4] and is regarded as the hallmark of epigenetic modification. DNA methylation occurs almost exclusively on cytosines within CpG dinucleotides, which are found in the genome at ~20% of the predicted frequency, and the

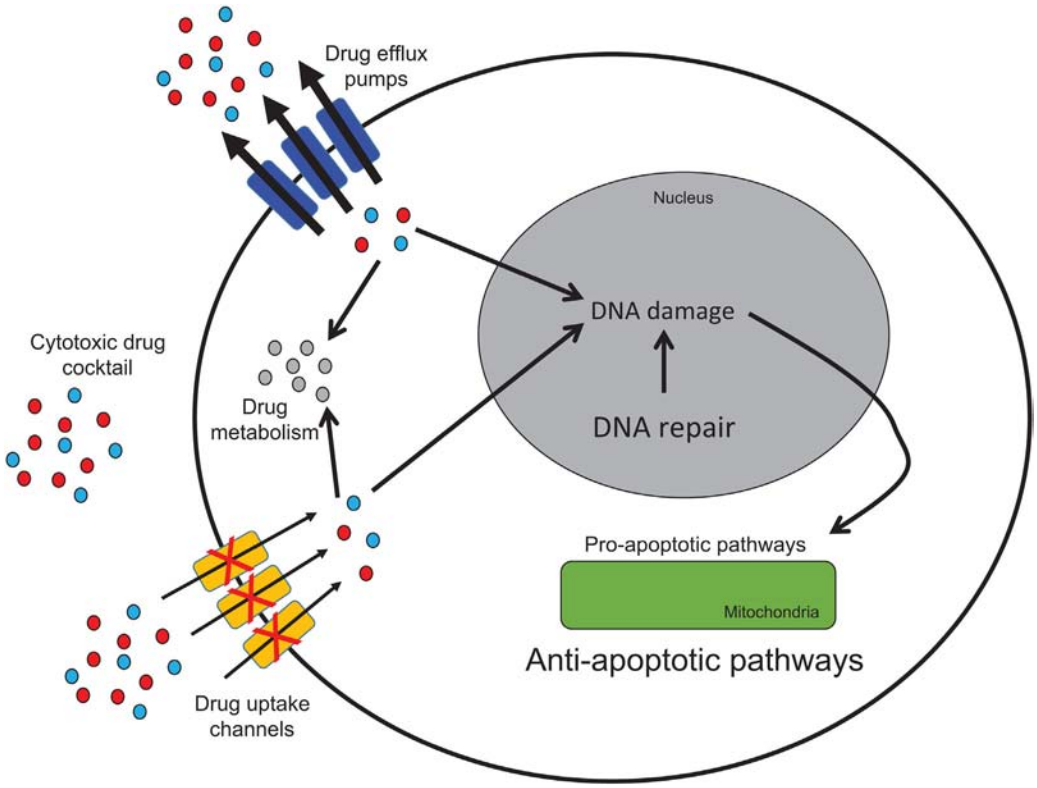

FIG. 9.2 **Cellular mechanisms of chemotherapy resistance.** Most cancer patients are treated with a cocktail of chemotherapeutic drugs (represented here by red and blue circles) which attack different pathways in the cancer cells. Cancer cells can avoid death in response to chemotherapy using various mechanisms. Shown here are decreased drug uptake, increased drug efflux, increased drug metabolism, increased DNA repair, and aberrant apoptotic mechanisms (decreased pro-apoptotic activity and increased anti-apoptotic activity).

majority (>70%) of CpG dinucleotides are typically methylated in any given cell type [5]. However, regions of CpG density, termed CpG islands [6,7], occur in the promoter sequences of many genes, proximal to their transcription start site [8]. CpG islands are conventionally defined as ≥200 bp with ≥50% G + C and ≥0.6 CpG observed/CpG expected [9], although a more rigorous definition (≥200 bp with ≥60% G + C and ≥0.7 CpG observed/CpG expected) has been proposed [7]. In reality, many investigators report detection of CpG islands in gene promoters that do not contain regions of CpG density conforming to these commonly accepted guidelines. However, good evidence has emerged that gene promoters with intermediate CpG features, or that lack CpG density, may be sensitive to and regulated by DNA methylation [10–12]. Gene promoters and other regulatory regions of housekeeping genes are largely unmethylated in most cell types, and tissue-specific genes are not methylated only in the cell types where these genes are transcriptionally active [13]. Most promoter CpG islands are unmethylated in normal tissues, but may become methylated in cancers leading

to transcriptional silencing of tumor suppressor genes (and other negative mediators of neoplastic growth) resulting in selective growth advantages for emergent neoplastic cells.

Neoplastic transformation is associated with alterations in DNA methylation, including both global hypomethylation and gene-specific hypermethylation [14–16]. DNA hypomethylation in cancer was first recognized in rodent models of liver cancer [17] and subsequently was reported in human cancer [18–20]. Hypomethylation of cancer cell genomes reflect loss of methylation in CpG-depleted regions where most CpG dinucleotides would be expected to be methylated [18,20,21]. The loss of methylation in these regions of the genome may be associated with aberrant or inappropriate expression of some genes that could contribute to neoplastic transformation, tumorigenesis, or cancer progression [19]. In addition, genome-wide demethylation has been shown to contribute to chromosomal instability by destabilizing pericentromeric regions of certain chromosomes [22–24]. Gains in DNA methylation in cancer cells typically reflect hypermethylation of CpG islands or other regions of CpG density in gene promoter regions, which can lead to gene silencing [14]. Methylation-dependent gene silencing is a normal mechanism for regulation of gene expression [25], but in cancer cells methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism for inactivation of tumor suppressor genes [26]. A significant number of cancer-related genes have been identified that are subject to methylation-dependent silencing [5,27] and many of these genes contribute to the hallmarks of cancer [28,29]. These observations combine to strongly suggest that epigenetic events, and particularly those involving DNA methylation, represent fundamental aspects of cancer and play key roles in neoplastic transformation and progression.

9.3 ABNORMAL DNA METHYLATION IN BREAST CANCER

Abnormal DNA methylation is a well-recognized hallmark of cancer [4,15,16,30]. DNA methylation-dependent epigenetic silencing of tumor suppressor genes and other genes required for neoplastic transformation, tumorigenesis, and tumor progression have been described in a number of studies. Recent evidence suggests that epigenetic mechanisms play a major role in breast carcinogenesis [10,31–37]. Epigenetic alterations differ from genetic alterations in that they occur more frequently, are reversible, and occur at defined regions of specific genes. DNA methylation is a well-known epigenetic mechanism, and a number of different genes have been shown to be inactivated in breast cancer through methylation-dependent gene silencing. Some of these genes are silenced through a direct effect of DNA methylation, whereas others are affected through indirect mechanisms.

Numerous methylation-sensitive genes have been identified in breast cancer cell lines through experimental studies [10,38], but not all have been functionally characterized. However, a number of important cancer-related genes are known to be epigenetically regulated in breast cancer. Among the genes that have been determined to be directly silenced by DNA methylation in breast cancer are cell-cycle control genes ($p16^{INK4a}$), steroid receptor genes (*ERα*, *PR*, *RARβ2*), tumor suppressor genes (*BRCA1*), genes associated with cancer metastasis (*E-cadherin*, *TIMP-3*), and others [39–43]. The $p16^{INK4a}$ cyclin-dependent kinase inhibitor is inactivated through methylation in several human cancers. In breast cancer, $p16^{INK4a}$ is methylated in 20%–30% of tumors and cell lines, with a concomitant loss of expression [44,45]. Loss of $p16^{INK4a}$ expression in this subset of breast cancers may contribute to unregulated cell proliferation and tumorigenesis. A significant percentage of breast cancers lack expression of

the estrogen receptor (and other steroid receptors), but loss of *ER* gene expression is not associated with gene deletion or somatic mutation [46]. Rather, methylation-dependent silencing of the *ER* gene is responsible for the loss of expression in these tumors [47,48]. Somatic mutations of the *BRCA1* gene do not frequently occur in nonhereditary breast cancers [49]. Therefore, an alternative mechanism for *BRCA1* inactivation involving DNA methylation was proposed [50–52]. Subsequently, several studies documented methylation-dependent epigenetic silencing of *BRCA1* in sporadic breast cancer [53–57]. Loss of *E-cadherin* gene expression in breast cancer is associated with an aggressive tumor phenotype and decreased patient survival [58]. Methylation-dependent loss of *E-cadherin* gene expression has been shown in 30% of primary breast cancers and up to 60% of metastatic tumors [59]. Loss of *TIMP-3* expression in breast tumors potentially results in increased proteolytic activity from matrix metalloproteinase enzymes [60]. The *TIMP-3* promoter is methylated in ~30% of primary breast cancers and breast cancer cell lines [61]. Both these methylation-related losses of gene expression are likely to contribute to tumor progression and spread.

9.4 EPIGENETIC THERAPY IN BREAST CANCER TREATMENT

Given that aberrant DNA methylation and epigenetic silencing of gene expression are now well-recognized hallmarks of cancer [4,15,16,30], numerous investigators have suggested that cancer should be treated with "epigenetic therapy" [62–64]. In contrast to gene therapy, epigenetic therapy (demethylating treatment) alters gene expression patterns in breast cancer without complications from enhanced immune response to therapeutic DNA. The reversibility of epigenetic alterations makes them excellent targets for improving breast cancer outcomes. The goal of such therapy would be to effect changes in gene expression, including re-expression of silenced genes (like tumor suppressor genes), that alter the clinical behavior of the tumor or the response of the tumor to other therapeutic modalities (such as chemotherapy). This concept has been tested in a breast cancer cell model system based on the MCF7 cell line using known demethylating drugs [65,66]. These studies provide strong evidence for enhancement of chemotherapeutic effect in MCF7 cells following demethylation of genomic DNA. These investigators have also initiated clinical trials with combination therapy using demethylating and cytotoxic drugs [67–69]. These studies strongly suggest that this epigenetic therapy will benefit breast cancer patients.

The majority of triple-negative breast cancers (basal-like and claudin-low breast cancers) overexpress DNMT3b, leading to aberrant DNA hypermethylation and concurrent loss of numerous methylation-sensitive genes [11,38]. It is likely that the methylation-dependent silencing of critical genes contributes significantly to the lack of sensitivity to standard chemotherapeutic agents by triple-negative breast cancers. The goal of epigenetic therapy in triple-negative breast cancers is to render cancer cells sensitive to chemotherapy through re-expression of methylation-sensitive genes secondary to normalization of DNA methylation patterns in response to inhibition of DNMT3b. The observation that primary breast cancers can be classified for aberrant DNA hypermethylation based upon methylation-sensitive gene expression signatures presents the opportunity to exploit gene-silencing events as biomarkers to identify which triple-negative breast cancer patients are most likely to benefit from epigenetic therapy [11].

Various forms of epigenetic therapy have been investigated experimentally. We explored pharmacologic epigenetic therapy and targeted epigenetic therapy in combination with cytotoxic chemotherapy in a breast cancer cell line model where the cell lines exhibit aberrant DNA hypermethylation related to overexpression of DNMT3b [70]. The goal of these studies was to evaluate the effectiveness of targeting the DNA methylation machinery to modify the sensitivity of breast cancer cells to cytotoxic drugs. Epigenetic treatment was accomplished through pharmacologic inhibition of DNA methyltransferase activity using 5-aza-2′-deoxycytidine (5-aza) and targeted inhibition of overexpressed DNMT3b using RNAi-mediated *DNMT3b* knockdown [70]. The results show that 5-aza pretreatment sensitizes breast cancer cells with aberrant DNA hypermethylation to cell killing by cytotoxic drugs and that the improved chemotherapeutic efficacy is a function of dose and duration of exposure to 5-aza [70]. Treatment of breast cancer cells for 7 days with low-dose 5-aza resulted in substantial decreases in the IC50 for doxorubicin (60%), paclitaxel (37%), and 5-fluorouracil (93%) [70].

Although we used 5-aza for these investigations, several other epigenetic drugs have been studied in a similar fashion [64,71–73]. In addition to DNMT inhibitors, histone deacetylase inhibitors may also prove to be useful in the epigenetic sensitization of breast cancer cells to chemotherapy, used alone or in conjunction with drugs like 5-aza. We also observed an increase in the effectiveness of chemotherapeutic drugs after targeted inhibition of DNMT3b using RNAi [70]. Although RNAi may not currently represent a viable approach for clinical treatment of breast cancer, model systems using RNAi in vitro demonstrate how targeted agents like small-molecule inhibitors might function therapeutically. Several small-molecule inhibitors of DNMT3b have been described [74]. Our results strongly suggest that DNMT3b is an excellent target for development of rational therapeutic approaches for breast cancers that exhibit aberrant DNA hypermethylation (such as triple-negative breast cancers). Our studies in cell lines provide proof-of-concept that targeting DNMT3b in breast cancer cells with aberrant DNA hypermethylation sensitizes them to cell killing by cytotoxic drugs and that this strategy can be exploited to improve patient outcomes that involve standard therapeutic approaches. Increasing the efficacy of chemotherapy through epigenetic therapy might benefit patients in at least two different ways. First, increasing the efficacy of a certain fixed dose of drug may increase the benefits of chemotherapy without associated increases in toxic side effects. Second, a lower dose of chemotherapeutic drug may be used to achieve a certain fixed therapeutic effect, but with diminished side effects.

Another approach to epigenetic therapy has emerged from our recent elucidation of the molecular basis of aberrant DNA hypermethylation in triple-negative breast cancers [75,76]. We have termed this approach biologic epigenetic therapy. Biologic epigenetic therapy is based upon the concept that normal regulation of DNMT3b can be restored by replacement of lost regulatory miRs [12]. Our experimental studies in cell lines showed that restoration of post-transcriptional regulation of DNMT3b mRNA can be accomplished using specific pre-miRs as single agents [75]. With recent development of nanoparticles for delivery of drugs and other active agents specifically to cancer cells, it becomes possible that pre-miRs might have practical utility as therapeutic agents. We would suggest that pre-miRs might be used in combination to target *DNMT3b* regulation in triple-negative breast cancers to normalize the methylome of these cancers and sensitize them to standard chemotherapies.

9.5 MECHANISMS GOVERNING IMPROVED RESPONSES TO CHEMOTHERAPY AFTER EPIGENETIC SENSITIZATION

Having made the observation that cell killing efficacy by chemotherapeutic drugs was enhanced following epigenetic drug exposure in breast cancer cell lines, the logical follow-up question revolves around the mechanism of sensitization. We speculated that exposure of cell lines to demethylating drugs restored expression of genes that are critical to pathways that produce cell death in response to cytotoxic drugs. Hence, pro-apoptotic and anti-apoptotic genes emerged as targets of interest. It is known that cancer cells avoid cell death through modification of apoptotic pathways.

We surveyed expression of 328 genes associated with apoptosis and survival in Hs578T breast cancer cells (which express the aberrant DNA hypermethylator phenotype) and identified several pro-apoptotic genes that are not expressed (undetected) or expressed at negligible levels in Hs578T cells, including *FASLG*, *IGF1*, *CD27*, and *BLK* (Table 9.1). Epigenetic silencing of these genes (directly or indirectly) would convey a survival advantage to the cancer cells through elimination of pro-apoptotic signaling (Fig. 9.3A). In addition, several members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamilies were identified that are not expressed (undetected) or expressed at negligible levels in Hs578T cells, including *TNFSF8*, *TNFSF10*, *TNFSF11*, *TNFSF14*, *TNFSF15*, *TNFRSF13B*, *TNFRSF17*, and *TNFRSF18* (Table 9.1). Among these TNF/TNFR superfamily genes, *TNFSF10*, *TNFSF14*, *TNFSF15*, *TNFRSF1B*, and *TNFRSF8* have been directly implicated as activators of apoptosis (Fig. 9.3B). Several of these pro-apoptotic genes and/or members of associated pathways have been shown to be subject to methylation-dependent silencing in cancer cells. In contrast, several anti-apoptotic genes were found to be abundantly expressed in Hs578T breast cancer cells, including *HSPB1* and *NPM1*. It is not known whether epigenetic mechanisms contribute to overexpression of anti-apoptotic genes in breast cancer. The contributions of aberrant DNA hypermethylation to the silencing of pro-apoptotic genes (and/or activation of anti-apoptotic genes) as a mechanism of chemotherapeutic resistance was explored further using a panel of breast cancer cell lines. Among the genes examined that regulate pro-apoptotic pathways, lack of expression was a consistent feature among breast cancer cell lines propagated in control medium (Fig. 9.4). However, significant induction of gene expression was observed for each of these genes in the index Hs578T breast cancer cell line following 5-aza treatment. In a similar fashion, substantial induction was seen in specific cell lines for specific genes in response to 5-aza treatment (Fig. 9.4). BLK is known to be methylation-sensitive, consistent with these responses to 5-aza treatment. These results suggest that FASLG, CD27, and IGF1 may also be subject to methylation-dependent silencing in breast cancer. Lack of expression of the genes that regulate pro-apoptotic pathways, either as a TNF signaling molecule (*TNFSF10*, *TNFSF11*, *TNFSF14*, *TNFSF15*) or as a TNFR molecule (*TNFRSF1B*, *TNFRSF8*, *TNFRSF13B*, *TNFRSF17*), was a consistent feature among breast cancer cell lines examined (Fig. 9.5). However, significant induction of gene expression was observed for each of these genes in the index Hs578T breast cancer cell line in response to 5-aza treatment. Likewise, substantial induction of specific TNF/TNFR superfamily genes was observed in response to 5-aza treatment of some breast cancer cell lines (Fig. 9.5). *TNFSF10*, *TNFRSF1B*, and *TNFRSF8* are known to be methylation-sensitive, consistent with the observed responses to 5-aza

TABLE 9.1 Discovery of Candidate Genes Related to Apoptosis and Survival in Human Hs578T Breast Cancer Cells

Genes not expressed		
ADCY1	Hs.192215	Adenylate cyclase 1
BCL2A1	Hs.227817	BCL2-related protein A1
BLK	Hs.146591	B lymphoid tyrosine kinase
CCL4	Hs.75703	Chemokine (C–C motif) ligand 4
CCL19	Hs.50002	Chemokine (C–C motif) ligand 29
CD27	Hs.355307	CD27 molecule
CR2	Hs.445757	Complement component receptor 2
CXCL13	Hs.100431	Chemokine (C–X–C motif) ligand 13
FASLG	Hs.2007	Fas ligand (TNF superfamily, member 6)
FCER2	Hs.465778	Fc fragment of IgE receptor, low-affinity II (CD23)
FLT3	Hs.507590	Fms-related tyrosine kinase 3
IGF1	Hs.160562	Insulin-like growth factor 1
IL-10	Hs.193717	Interleukin 10
IL-2	Hs.89679	Interleukin 2
INPP5D	Hs.262886	Inositol polyphosphate-5-phosphatase
NOS1	Hs.654410	Nitric oxide synthase 1
NOS3	Hs.647092	Nitric oxide synthase 3
PRKACG	Hs.158029	Protein kinase, cAMP-dependent, catalytic, gamma
TNFSF10	Hs.478275	Tumor necrosis factor superfamily, member 10
TNFSF11	Hs.333791	Tumor necrosis factor superfamily, member 11
TNFSF14	Hs.129708	Tumor necrosis factor superfamily, member 14
TNFSF15	Hs.23349	Tumor necrosis factor superfamily, member 15
TNFRSF1B	Hs.256278	Tumor necrosis factor receptor superfamily, member 1B
TNFRSF8	Hs.1314	Tumor necrosis factor receptor superfamily, member 8
TNFRSF13B	Hs.158341	Tumor necrosis factor receptor superfamily, member 13B
TNFRSF17	Hs.2556	Tumor necrosis factor receptor superfamily, member 17
VAV1	Hs.116237	Vav 1 guanine nucleotide exchange factor
Genes expressed at low levels		
CCL21	Hs.57907	Chemokine (C–C motif) ligand 21
CD40LG	Hs.592244	CD40 ligand
PRKCZ	Hs.496255	Protein kinase C, zeta
TNFSF8	Hs.494901	Tumor necrosis factor superfamily, member 8
Genes expressed at high levels		
GNAS	Hs.125898	GNAS complex locus
HSPB1	Hs.520973	Heat shock 27 kDa protein 1
NPM1	Hs.557550	Nucleophosmin
PPIB	Hs.434937	Peptidylprolyl isomerase B
SQSTM1	Hs.724025	Sequestosome 1

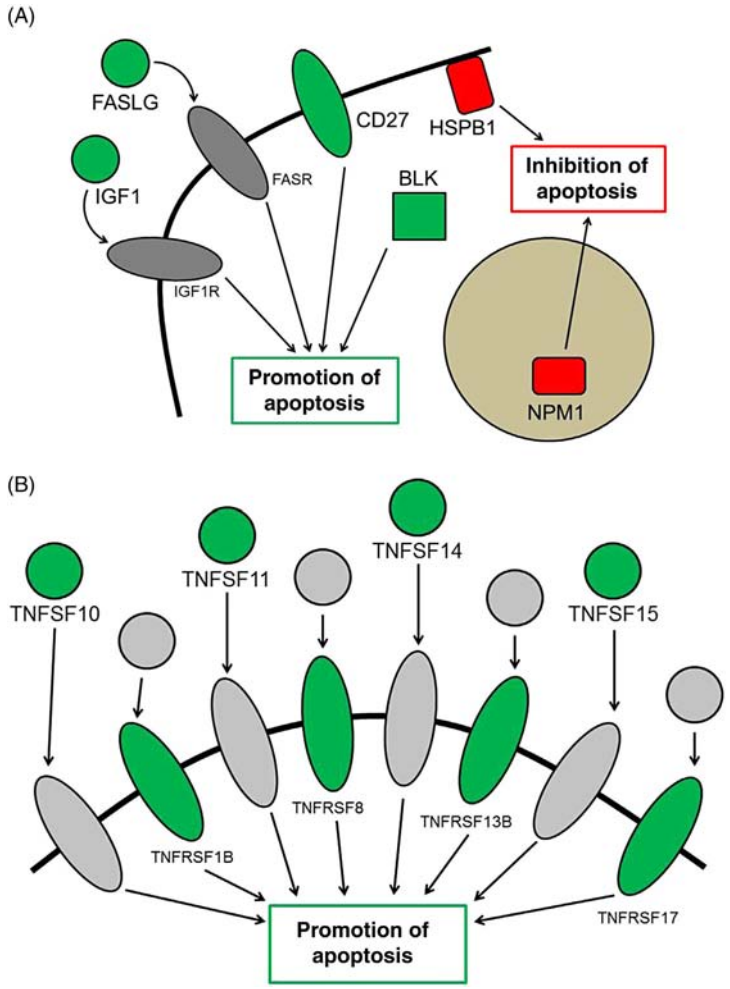

FIG. 9.3 **Pro-apoptotic and TNF superfamily genes.** (A) The response of four genes directly involved in pro-apoptotic pathways to demethylating drug treatment was examined, including cell-surface receptor CD27 (TNFRSF7), ligands for FASR (FASLG) and IGF1R (IGF1), and cytoplasmic tyrosine kinase (BLK). The protein products for these genes are shown in green in the schematic. Two genes that inhibit apoptosis, including HSPB1 (HSP27) and NPM1, are described in the text. The protein products for these genes are shown in red in the schematic. (B) The response of eight TNF/TNFR superfamily genes to demethylating drug treatment was examined. The protein products of these genes are directly involved in pro-apoptotic pathways, including TNF ligands (TNFSF10, TNFSF11, TNFSF14, TNFSF15), and cell-surface TNF receptors (TNFRSF1B, TNFRSF8, TNFRSF13B, TNFRSF17). The protein products for these genes are shown in green in the schematic. Abbreviation: TNF, tumor necrosis factor.

treatment. These results suggest that *TNFSF11*, *TNFSF14*, *TNFSF15*, *TNFRSF13B*, and *TNFRSF17* may also be subject to methylation-dependent silencing in breast cancer. Further studies are needed to examine promoter methylation events in these pro-apoptotic and TNF/TNFR superfamily genes in breast cancer cells that display the aberrant DNA hypermethylation phenotype and in response to demethylating drugs like 5-aza.

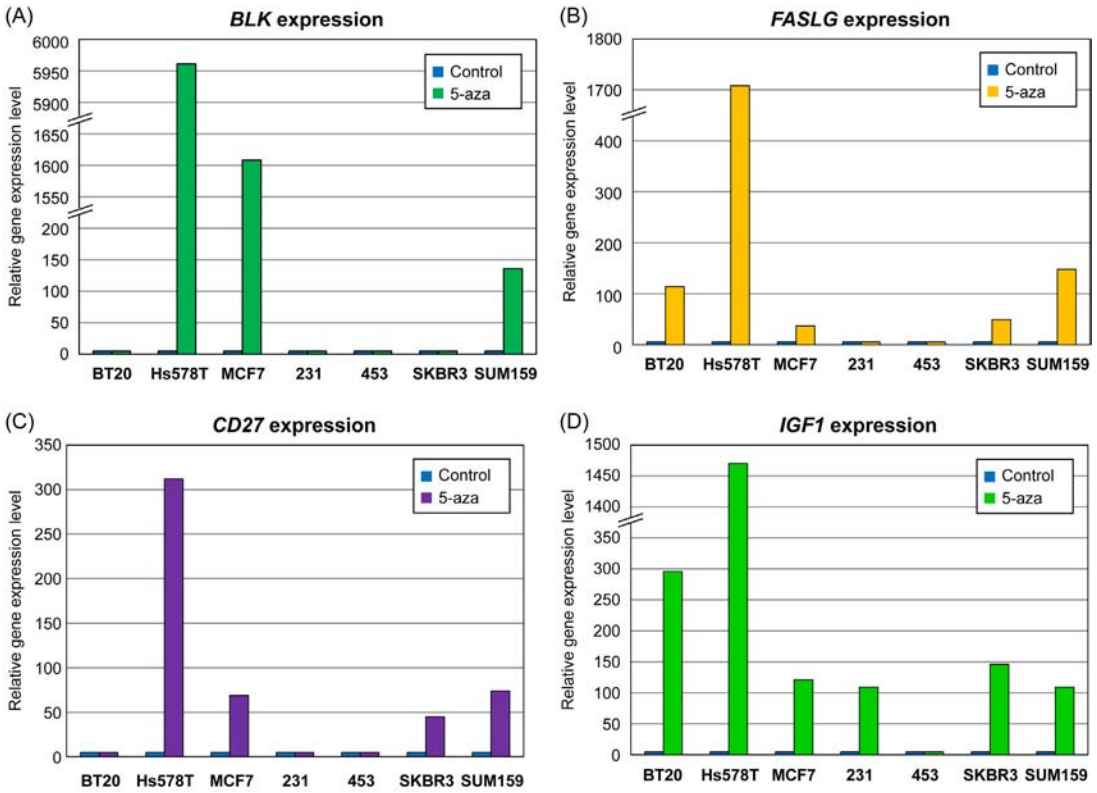

FIG. 9.4 **Re-expression of pro-apoptotic genes in breast cancer cell lines following treatment with demethylating drugs.** A panel of human breast cancer cell lines consisting of BT20, Hs578T, MCF7, MDA-MB-231 (represented as 231), MDA-MB-453 (represented as 453), SKBR3, and SUM159 were propagated in DMEM/F12 mix medium containing 10% fetal calf serum with or without 500 nM 5-aza-2′-deoxycytidine for 7 days [70]. At the end of the culture period, cells were harvested, RNA prepared, and real-time PCR was conducted for the genes shown: (A) *BLK*; (B) *FASLG*; (C) *CD27*; (D) *IGF1*.

9.6 EPIGENETICS OF CHEMORESISTANCE

The studies reviewed here suggest strongly that re-expression of genes associated with pro-apoptotic pathways in response to demethylating drugs represents one mechanism for sensitizing breast cancer cells to cytotoxic chemotherapy. However, additional investigations are warranted to determine whether re-expression of pro-apoptotic genes is the direct result of demethylating events in their associated promoters, or whether indirect mechanisms account for some of these responses. With ever-broadening studies of the human epigenome (and various cancer epigenomes), important information related to mechanisms of chemotherapy resistance and sensitization will be gained. Likewise, as greater understanding of these phenomena is gained, appropriate strategies can be developed to address epigenetic therapy of human cancer in the clinical setting. Possibilities include the use of broad-spectrum demethylating drugs (like 5-aza) or novel targeted drugs (like

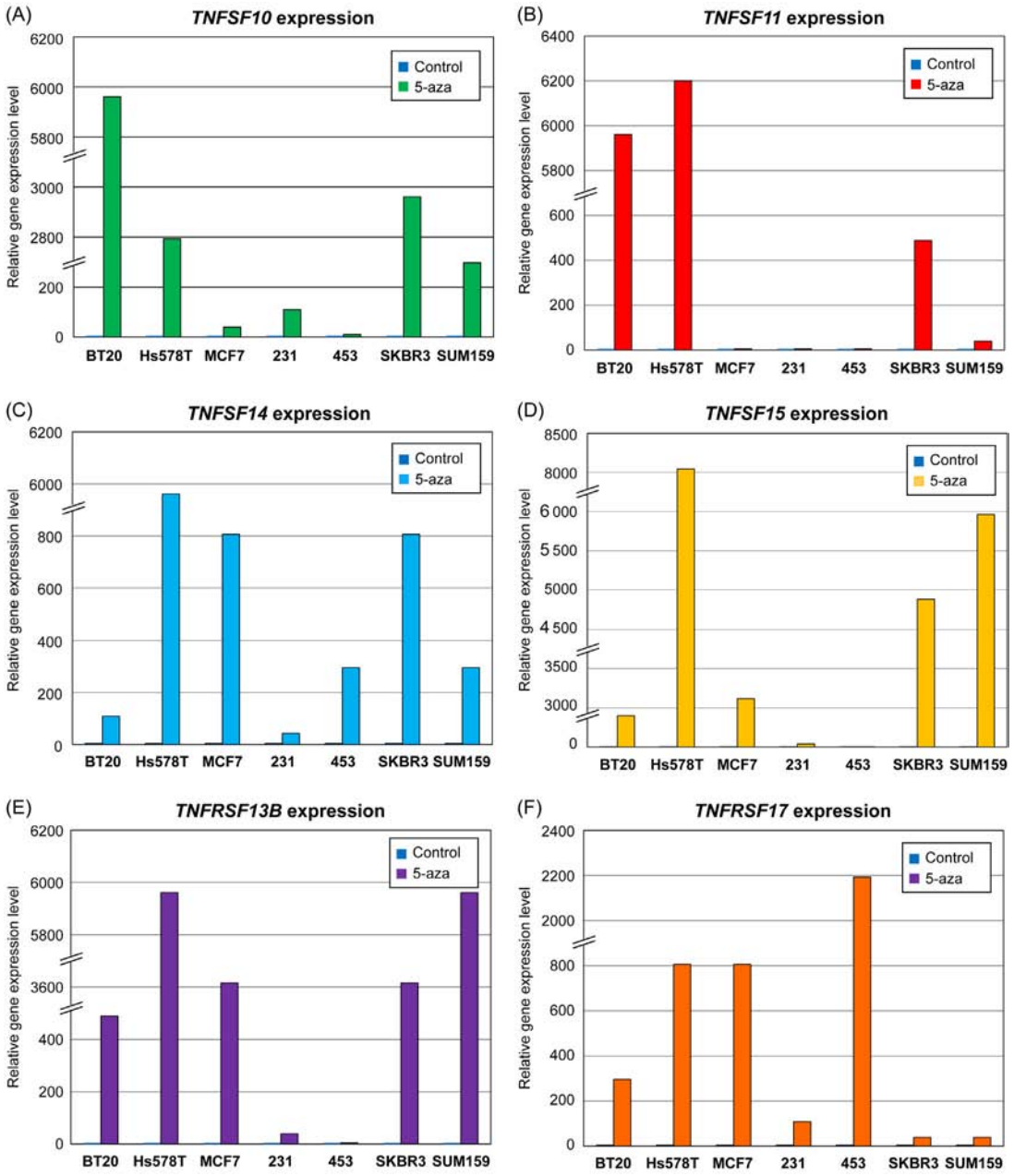

FIG. 9.5 **Re-expression of TNF and TNFR genes in breast cancer cell lines following treatment with demethylating drugs.** A panel of human breast cancer cell lines consisting of BT20, Hs578T, MCF7, MDA-MB-231 (represented as 231), MDA-MB-453 (represented as 453), SKBR3, and SUM159 were propagated in DMEM/F12 mix medium containing 10% fetal calf serum with or without 500 nM 5-aza-2′-deoxycytidine for 7 days [70]. At the end of the culture period, cells were harvested, RNA prepared, and real-time PCR was conducted for the genes shown: (A) *TNFSF10*; (B) *TNFSF11*; (C) *TNFSF14*; (D) *TNFSF15*; (E) *TNFRSF13B*; (F) *TNFRSF17*.

small-molecule inhibitors) that correct the aberrant methylome of cancer cells resulting in sensitization to cytotoxic drugs (or other therapeutic modalities such as immunotherapy or other targeted therapies).

9.7 EPIGENETIC CANCER THERAPY

The ultimate goal of epigenetic therapy in cancer is to reverse the biology associated with the malignant phenotype associated with aberrant DNA hypermethylation events (and/or changes in histone modifications), which results in changes in gene expression patterns. The changes in gene expression patterns observed with demethylating treatment are complex and extend beyond upregulation of a subset of genes due to promoter demethylation [10,12]. Of importance, some genes show downregulation in response to demethylating drug treatment, which might be due to direct mechanisms or indirect mechanisms. The response to demethylating drugs will require much more study before all of these molecular outcomes are known.

There are several possible beneficial outcomes of epigenetic therapy in cancer. One of these is the sensitization of the cancer cells to traditional cytotoxic chemotherapy, to enhance cell killing or to overcome resistance. This has been shown experimentally to be a plausible goal with respect to the use of demethylating drugs. In some cancers, re-expression of critical genes may result in a slowing of cell proliferation or loss of viability of the tumor-initiating cells (perhaps related to re-expression of a tumor suppressor gene or pathway). Loss of proliferative ability of the cancer in response to tumor suppressor gene expression might reflect an exit from the cell cycle (movement toward differentiation) or activation of some SOS system and cell death. All of these possibilities could improve outcomes for cancer patients. However, there are a number of unanswered questions. For instance, will epigenetic sensitization of cancer cells result in a sensitization of the host? This could result in a greater magnitude of cytotoxic effects on the bone marrow and other critical systems in the body and would be counterproductive to therapy (especially if the cytotoxic drug dose needed to be reduced). Experimental studies in animals will address some of these questions effectively if properly designed and controlled. Likewise, carefully planned human studies could also answer some of these critical questions prior to wide application of epigenetic drugs in the clinical setting in routine oncology practice.

Conflict of Interest: No potential conflicts of interest were disclosed.

References

[1] DeVita VT Jr, Chu E. A history of cancer chemotherapy. Cancer Res 2008;68(21):8643–53.
[2] Siegel RL, Miller KD, Jemal A. Cancer statistics. Cancer J Clin 2017;67(1):7–30.
[3] Feinberg AP. The epigenetics of cancer etiology. Semin Cancer Biol 2004;14(6):427–32.
[4] Baylin SB. DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2005;2(Suppl. 1):S4–S11.
[5] Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene 2002;21(35):5462–82.
[6] Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068–70.
[7] Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 2002;99:3740–5.
[8] Bird AP. CpG-rich islands and the function of DNA methylation. Nature 1986;321:209–13.
[9] Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987;196(2):261–82.
[10] Rivenbark AG, Jones WD, Risher JD, Coleman WB. DNA methylation-dependent epigenetic regulation of gene expression in MCF-7 breast cancer cells. Epigenetics 2006;1(1):32–44.

[11] Roll JD, Rivenbark AG, Sandhu R, Parker JS, Jones WD, Carey LA, et al. Dysregulation of the epigenome in triple-negative breast cancers: basal-like and claudin-low breast cancers express aberrant DNA hypermethylation. Exp Mol Pathol 2013;95(3):276–87.
[12] Sandhu R, Roll JD, Rivenbark AG, Coleman WB. Dysregulation of the epigenome in human breast cancer: contributions of gene-specific DNA hypermethylation to breast cancer pathobiology and targeting the breast cancer methylome for improved therapy. Am J Pathol 2015;185(2):282–92.
[13] Paulsen M, Tierling S, Walter J. DNA methylation and the mammalian genome. In: Tost J, editor. Epigenetics. Norfolk, UK: Caister Academic Press; 2008. p. 1–21.
[14] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349(21):2042–54.
[15] Baylin S. DNA methylation and epigenetic mechanisms of carcinogenesis. Dev Biol (Basel) 2001;106:85–7.
[16] Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–96.
[17] Lapeyre JN, Becker FF. 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogenesis and in carcinomas which result. Biochem Biophys Res Commun 1979;87(3):698–705.
[18] Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983;301(5895):89–92.
[19] Feinberg AP, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. Biochem Biophys Res Commun 1983;111(1):47–54.
[20] Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 1985;228(4696):187–90.
[21] Feinberg AP, Vogelstein B. Alterations in DNA methylation in human colon neoplasia. Semin Surg Oncol 1987;3(3):149–51.
[22] Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 1998;77(6):833–8.
[23] Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science 2003;300(5618):455.
[24] Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300(5618):489–92.
[25] Momparler RL. Cancer epigenetics. Oncogene 2003;22(42):6479–83.
[26] Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21(2):163–7.
[27] Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. Oncogene 2002;21(35):5450–61.
[28] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57–70.
[29] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646–74.
[30] Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10(7):687–92.
[31] Bae YK, Brown A, Garrett E, Bornman D, Fackler MJ, Sukumar S, et al. Hypermethylation in histologically distinct classes of breast cancer. Clin Cancer Res 2004;10(18 Pt 1):5998–6005.
[32] Domann FE, Rice JC, Hendrix MJ, Futscher BW. Epigenetic silencing of maspin gene expression in human breast cancers. Int J Cancer 2000;85(6):805–10.
[33] Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in *in situ* and invasive lobular breast carcinoma. Int J Cancer 2003;107(6):970–5.
[34] Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. Hum Mol Genet 1999;8(3):459–70.
[35] Jhaveri MS, Morrow CS. Methylation-mediated regulation of the glutathione S-transferase P1 gene in human breast cancer cells. Gene 1998;210(1):1–7.
[36] Rivenbark AG, Jones WD, Coleman WB. DNA methylation-dependent silencing of CST6 in human breast cancer cell lines. Lab Invest 2006;86(12):1233–42.
[37] Rivenbark AG, Livasy CA, Boyd CE, Keppler D, Coleman WB. Methylation-dependent silencing of CST6 in primary human breast tumors and metastatic lesions. Exp Mol Pathol 2007;83(2):188–97.
[38] Roll JD, Rivenbark AG, Jones WD, Coleman WB. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer 2008;7:15.
[39] Yang X, Yan L, Davidson NE. DNA methylation in breast cancer. Endocr Relat Cancer 2001;8(2):115–27.

[40] Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, et al. Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 2003;63(19):6178–86.
[41] Dammann R, Yang G, Pfeifer GP. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 2001;61(7):3105–9.
[42] Widschwendter M, Berger J, Muller HM, Zeimet AG, Marth C. Epigenetic downregulation of the retinoic acid receptor-beta2 gene in breast cancer. J Mammary Gland Biol Neoplasia 2001;6(2):193–201.
[43] Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst 2000;92(10):826–32.
[44] Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995;55(20):4525–30.
[45] Woodcock DM, Linsenmeyer ME, Doherty JP, Warren WD. DNA methylation in the promoter region of the p16 (CDKN2/MTS-1/INK4A) gene in human breast tumours. Br J Cancer 1999;79(2):251–6.
[46] Lapidus RG, Nass SJ, Davidson NE. The loss of estrogen and progesterone receptor gene expression in human breast cancer. J Mammary Gland Biol Neoplasia 1998;3(1):85–94.
[47] Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 1994;54(10): 2552–5.
[48] Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, et al. Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 1996;2(5):805–10.
[49] Merajver SD, Pham TM, Caduff RF, Chen M, Poy EL, Cooney KA, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. Nat Genet 1995;9(4):439–43.
[50] Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res 1997;57(16):3347–50.
[51] Magdinier F, Ribieras S, Lenoir GM, Frappart L, Dante R. Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. Oncogene 1998;17(24):3169–76.
[52] Rice JC, Massey-Brown KS, Futscher BW. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene 1998;17(14):1807–12.
[53] Bianco T, Chenevix-Trench G, Walsh DC, Cooper JE, Dobrovic A. Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis 2000;21(2):147–51.
[54] Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene 1999;18(11):1957–65.
[55] Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001;344(8):539–48.
[56] Magdinier F, Billard LM, Wittmann G, Frappart L, Benchaib M, Lenoir GM, et al. Regional methylation of the 5′ end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. FASEB J 2000;14(11):1585–94.
[57] Miyamoto K, Fukutomi T, Asada K, Wakazono K, Tsuda H, Asahara T, et al. Promoter hypermethylation and post-transcriptional mechanisms for reduced BRCA1 immunoreactivity in sporadic human breast cancers. Jpn J Clin Oncol 2002;32(3):79–84.
[58] Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM, Schalken JA. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. Cancer Res 1993;53(14):3241–5.
[59] Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE, et al. Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. Cancer Res 2000;60(16):4346–8.
[60] Uria JA, Ferrando AA, Velasco G, Freije JM, Lopez-Otin C. Structure and expression in breast tumors of human TIMP-3, a new member of the metalloproteinase inhibitor family. Cancer Res 1994;54(8):2091–4.
[61] Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res 1999;59(4):798–802.
[62] Brueckner B, Kuck D, Lyko F. DNA methyltransferase inhibitors for cancer therapy. Cancer J 2007;13(1):17–22.

[63] Brueckner B, Lyko F. DNA methyltransferase inhibitors: old and new drugs for an epigenetic cancer therapy. Trends Pharmacol Sci 2004;25(11):551–4.
[64] Yoo CB, Cheng JC, Jones PA. Zebularine: a new drug for epigenetic therapy. Biochem Soc Trans 2004;32(Pt 6): 910–2.
[65] Segura-Pacheco B, Perez-Cardenas E, Taja-Chayeb L, Chavez-Blanco A, Revilla-Vazquez A, Benitez-Bribiesca L, et al. Global DNA hypermethylation-associated cancer chemotherapy resistance and its reversion with the demethylating agent hydralazine. J Transl Med 2006;4:32.
[66] Chavez-Blanco A, Perez-Plasencia C, Perez-Cardenas E, Carrasco-Legleu C, Rangel-Lopez E, Segura-Pacheco B, et al. Antineoplastic effects of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid in cancer cell lines. Cancer Cell Int 2006;6:2.
[67] Zambrano P, Segura-Pacheco B, Perez-Cardenas E, Cetina L, Revilla-Vazquez A, Taja-Chayeb L, et al. A phase I study of hydralazine to demethylate and reactivate the expression of tumor suppressor genes. BMC Cancer 2005;5(1):44.
[68] Candelaria M, Gallardo-Rincon D, Arce C, Cetina L, Aguilar-Ponce J, Arrieta O, et al. A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. Ann Oncol 2007;18(9):1529–38.
[69] Arce C, Perez-Plasencia C, Gonzalez-Fierro A, de la Cruz-Hernandez E, Revilla-Vazquez A, Chavez-Blanco A, et al. A proof-of-principle study of epigenetic therapy added to neoadjuvant doxorubicin cyclophosphamide for locally advanced breast cancer. PLoS ONE 2006;1:e98.
[70] Sandhu R, Rivenbark AG, Coleman WB. Enhancement of chemotherapeutic efficacy in hypermethylator breast cancer cells through targeted and pharmacologic inhibition of DNMT3b. Breast Cancer Res Treat 2012;131(2):385–99.
[71] Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebulariwne. J Natl Cancer Inst 2003;95(5):399–409.
[72] Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, et al. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. Mol Cancer Ther 2005;4(10):1515–20.
[73] Marquez VE, Kelley JA, Agbaria R, Ben-Kasus T, Cheng JC, Yoo CB, et al. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. Ann NY Acad Sci 2005;1058:246–54.
[74] Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. J Natl Cancer Inst 2005;97(20):1498–506.
[75] Sandhu R, Rivenbark AG, Coleman WB. Loss of post-transcriptional regulation of DNMT3b by microRNAs: a possible molecular mechanism for the hypermethylation defect observed in a subset of breast cancer cell lines. Int J Oncol 2012;41(2):721–32.
[76] Sandhu R, Rivenbark AG, Mackler RM, Livasy CA, Coleman WB. Dysregulation of microRNA expression drives aberrant DNA hypermethylation in basal-like breast cancer. Int J Oncol 2014;44(2):563–72.