ELSEVIER

Breast Cancer Theme Issue

The American Journal of
PATHOLOGY

ajp.amjpathol.org

REVIEW

The Epigenomic Revolution in Breast Cancer

From Single-Gene to Genome-Wide Next-Generation Approaches

Veronica Davalos,^{*} Anna Martinez-Cardus,^{*} and Manel Esteller^{*†‡}

From the Cancer Epigenetics and Biology Program,^{*} Bellvitge Biomedical Research Institute, Barcelona; the Department of Physiological Sciences II,[†] School of Medicine, University of Barcelona, Barcelona; and the Institutio Catalana de Recerca i Estudis Avançats,[‡] Barcelona, Spain

Accepted for publication
July 6, 2017.

Address correspondence to
Manel Esteller, M.D., Ph.D.,
Cancer Epigenetics and Biology
Program (PEBC), Hospital
Duran i Reynals, 3a planta -
Gran Via de L'Hospitalet, 199,
08908 L'Hospitalet de Llobregat,
Barcelona, Spain.
E-mail: mesteller@idibell.cat.

From the first identification of aberrant DNA methylation in primary tumors in humans more than 3 decades ago, progress in cancer epigenetics research has been exponential. For many years, cancer epigenetics studies relied on the identification of DNA methylation and histone modifications at specific genes. Those studies laid the foundation for the field and revealed the epigenetic alterations as hallmarks of cancer, as well as the crucial role of epigenetic mechanisms in tumorigenesis. The introduction of next-generation sequencing and array-based technologies for analyzing epigenetic states has accelerated our understanding about cancer and have become potent tools in our fight against the disease. Findings emerging from epigenome-wide analyses have led to new discoveries with remarkable clinical applications. This review summarizes the gene-specific epigenetic alterations common in breast cancer and discusses the recent advances in breast cancer epigenomics, focusing on their contribution to diagnosis, prognosis, patient stratification, and treatment of the disease. (*Am J Pathol* 2017, 187: 2163–2174; <http://dx.doi.org/10.1016/j.ajpath.2017.07.002>)

The term *epigenetics* was introduced by C.H. Waddington in 1942 to refer to the study of the processes by which genotype give rise to phenotype.¹ It was later redefined as those heritable changes in gene expression that do not involve changes in the underlying DNA sequence.² The most studied epigenetic modifications include DNA methylation and histone changes. These modifications modulate chromatin conformation and have crucial roles in the regulation of gene expression and in the accessibility of transcription factors, coactivators, and co-repressors.

In humans, DNA methylation occurs at the carbon-5 of the cytosine in 5'-C-phosphate-G-3' (CpG) dinucleotides. This reaction is catalyzed by DNA methyltransferases (DNMTs). DNMTs 3a and 3b (*de novo* DNMTs) transfer a methyl group from S-adenosyl-methionine to previously unmethylated cytosines, whereas DNMT1 (maintenance DNMT) preserves the methylation patterns throughout each cell division. CpG sites are not randomly distributed in the

genome, but instead there are CpG-rich zones known as *CpG islands*, located mainly at the regulatory regions in 40% to 60% of all genes.^{3,4} Methylation of CpG islands is a rare event in normal cells, restricted to untranscribed genes in the X chromosome, imprinted genes, germline genes, and some tissue-specific genes. However, CpG island

Supported by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7-PEOPLE-2013-IOF) REA grant agreement No. 623443. (V.D.). M.E. is an Institutio Catalana de Recerca i Estudis Avançats Research Professor. M.E.'s research group receives funding from Instituto de Salud Carlos III, cofinanced by the A Way to Achieve Europe European Development Regional Fund under the TRANSCAN ERA-NET on Translational Cancer Research grants AC14/00023, CANCER13-FP-011, and CIBERONC CB16/12/00312.

Disclosures: None declared.

This article is part of a review series on next-generation breast cancer omics.

hypermethylation is a common hallmark in cancer cells, first associated with tumor suppressor gene silencing.⁴

Along with DNA methylation, histone modifications are the most studied epigenetic events related to cancer progression. Regulation of gene expression can occur through post-translational modifications of the histone tails, including covalent changes such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation (small ubiquitin-like modifiers), proline isomerization, and ADP-ribosylation. Their presence on histones form the so-called *histone code*, which dictates the chromatin structure in which DNA is packaged and can orchestrate the ordered recruitment of enzyme complexes to wrap the DNA.^{5,6} This histone code is written and erased by histone modifying enzymes. The *writer* of histone modification refers to an enzyme that catalyzes a chemical modification of histones in a residue-specific manner (ie, histone lysine methyltransferases or histone lysine acetyltransferases), and the *eraser* of histone marks refers to an enzyme that removes a chemical modification from histones [ie, histone lysine demethylases or histone lysine deacetylases (HDAC/KDAC)]. This code is interpreted by reader or effector proteins that specifically bind to a certain type or a combination of histone modifications and translate the histone code into a meaningful biological outcome, whether it is transcriptional activation or silencing, or other cellular responses. In addition to this recruitment mechanism, histone marks *per se* can modulate the chromatin conformation based on steric or charge interactions (ie, neutralization of the positive charges of histones by acetylation of lysines).^{5–7} Altogether, this complex epigenetic network guarantees dynamic and accurate control of gene expression.

From the first identification of aberrant DNA methylation in primary human tumors more than 3 decades ago,⁸ the epigenetics field has achieved a position as relevant as genetics in cancer research. Numerous studies identifying gene-specific epigenetic alterations that occur in cancer have revealed that in addition to genetic aberrations, epigenetic alterations are crucial players in cancer development and progression. In the past few years, the introduction of next-generation sequencing and array-based technologies has accelerated and expanded our knowledge about the relevance of epigenetic mechanisms in tumorigenesis. Moreover, the clinical applications of findings from epigenome-wide analyses are increasing. In this review, we provide an overview of gene-specific epigenetic alterations common in breast cancer and discuss the recent advances in breast cancer epigenomics, focusing on their contribution to diagnosis, prognosis, patient stratification, and treatment of the disease.

Gene-Specific Epigenetic Alterations in Breast Cancer

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related death among

women worldwide. In United States, approximately 255,180 new cases of breast carcinoma will be diagnosed and 41,070 deaths will occur during 2017.⁹ Gene expression profiling studies have classified breast cancers into subtypes with different prognostic behavior.¹⁰ Most breast tumors are characterized by the expression of estrogen receptor α (*ESR1*), progesterone receptor (*PR*), or human epidermal growth factor receptor 2 [*HER2* (alias *ERBB2*)] proto-oncogene. Treatment with antiestrogens and agents targeting *HER2* significantly improve survival in many of these cases.¹¹ However, there is a subset of 15% to 25% of breast cancer cases that are negative for *ESR1*, *PR*, and *HER2*, called triple-negative breast cancers (TNBCs), in which chemotherapy-based schedules are the standard of care, in part due to the lack of target-specific therapies, making it the subtype associated with worse prognosis.¹¹

Despite it being shown that hormone receptor positivity is predictive of response to antiestrogen treatment, 30% to 40% of patients develop resistance. The loss of estrogen receptor (*ER*) expression increases tumor aggressiveness and leads to failure of endocrine therapy.¹² Moreover, some tumors *ER*-positive (*ER*⁺) at the time of diagnosis become *ER*-negative (*ER*[−]) in the course of the clinical evolution of the disease. Interestingly, whereas hypermethylation of neither *ESR1* nor *PR* seems to be a good predictor of its own receptor status, each is the best predictor of the status of the other receptor.^{13,14} Thus, a lack of methylation of *ESR1* is associated with *PR* expression, as activated *ER* induces *PR* expression. Widschwendter et al¹³ found that *ESR1* methylation is a good survival predictor only in tamoxifen-treated patients, whereas *ARHI* methylation predicts survival only in non-tamoxifen-treated patients. Moreover, they showed that *CYP1B1* methylation predicts tamoxifen response. Additionally, Martínez-Galán et al¹⁵ observed a significant correlation between the hypermethylation of *ESR1* in the peripheral blood and lack of *ER* expression in excised tumor tissue. They suggested a prognostic role of *ESR1* methylation status in the identification of luminal phenotypes with poor prognosis and potentially higher resistance to hormone treatment. Additionally, the direct relationship described between *RASSF1A* promoter methylation and the expression of *ER* could also add value in the prognosis of hormone therapy response.¹⁶

On the other hand, triple-negative tumors, characterized by a lack of expression of *ESR1*, *PR*, and *HER2*, show an enrichment of alterations in DNA repair genes, mainly *BRCA1*.¹⁰ *BRCA* proteins 1 and 2 are involved in double-strand break repair through homologous recombination.¹⁷ Germline mutations in the *BRCA* genes may be involved in up to 30% of inherited breast carcinomas.⁹ However, in sporadic disease, silencing of *BRCA1* largely occurs by promoter hypermethylation, especially in the presence of loss of heterozygosity and in the triple-negative subgroup.^{18,19} These potentially lethal events in homologous recombination-deficient cells can be exploited for

therapeutic purposes.^{17–20} For instance, the effect of *BRCA1* hypermethylation in sensitivity to DNA-damaging chemotherapeutic agents such as cisplatin in TNBC patients has been described.²¹ Interestingly, Veeck et al¹⁹ demonstrated that *BRCA1* promoter hypermethylation predicted sensitivity to poly(ADP-ribose) polymerase (PARP) inhibitors in nonhereditary TNBC, suggesting its inclusion in prospective clinical trials as a potential biomarker of sensitivity to these compounds. The mechanism through which *BRCA1* methylation sensitizes tumors to PARP inhibitors is described in Figure 1. Nonetheless, *BRCA1* re-expression with retained *BRCA1* promoter hypermethylation has been detected in patient-derived xenografts of *BRCA1*-deficient breast cancer. *De novo* gene fusions place *BRCA1* under the transcriptional control of a heterologous promoter, resulting in re-expression of *BRCA1* and the acquisition of therapy resistance.²²

Furthermore, epigenetic alterations in cancer-related pathways in *HER2*⁺ breast cancers have also been identified, such as the activation of the wingless-type mouse mammary tumor virus integration site family (WNT) pathway by aberrant methylation of its negative regulators.²³ Hypermethylation-mediated silencing of the genes encoding the WNT-negative regulators *WIF1* and *DKK3* has been reported in breast carcinogenesis.^{24–26}

The DNA-methylation status of a different panel of genes associated with cellular processes potentially involved in

breast carcinogenesis has been widely studied. Fackler et al²⁷ reported significant hypermethylation of *TWIST*, *RASSF1A*, *CCND2*, and *HIN1* in breast tumor samples but not in normal tissue. Afterward, these observations were corroborated in other similar works: Jeronimo et al²⁸ described the potential roles of *RASSF1A*, *CCND2*, *HIN1*, and *APC* as diagnostic biomarkers, using quantitative methylation-specific PCR in fine-needle aspiration breast biopsies; other investigators have found that hypermethylation of *RASSF1A*, *CCND2*, *HIN1*, and *APC* was correlated with hormone receptor (HR)⁺ breast cancer phenotypes, with lower frequencies in ER and *HER2* double-negative tumors.^{29,30} Some reports have also shown that hypermethylation of *CDH13* and *GSTP1* occurs more frequently in triple-negative and lymph nodes-positive patients, respectively.^{13,29–31}

Radpour et al³² determined, by semiquantitative methylation changes through mass spectrometry-based techniques, a set of hypermethylated genes associated with breast carcinogenesis: In addition to the aforementioned *BRCA1*, *APC*, and *GSTP1*, they found *BIN1*, *BMP6*, *ESR2*, *CDKN2A* (p16), *CDKN1A* (p21), *TIMP3*, and *CST6*. Interestingly, hypermethylation of the *CST6* promoter has also been detected in cell-free DNA, showing its value as a prognostic factor of relapse and of survival in operable breast cancer patients.³³ Hypermethylation of other gene promoters detected in circulating tumor cells and cell-free

Figure 1 *BRCA1* methylation sensitizes to poly(ADP-ribose) polymerase inhibitors (PARPi). On DNA damage, PARP1 is recruited to the site of the single-strand break, where it catalyzes a series of PARylation events, leading to the DNA repair process. PARP inhibitors prevent the release of PARP1 from DNA. During DNA replication, these single-strand breaks turn into stalled replication forks that are repaired by DNA-replication-dependent homologous recombination repair mechanisms, in which *BRCA1* protein plays an essential role. *BRCA1* 5'-C-phosphate-G-3' (CpG) island hypermethylation-dependent silencing, which results in lack of *BRCA1* protein, impairs this mechanism. In *BRCA1*-deficient cells, 53BP1 occupies sites of damage, promoting error-prone DNA repair mechanisms such as nonhomologous end joining (NHEJ), leading to chromosome rearrangements. Genomic instability and the accumulation of stalled replication forks generate cytotoxicity, resulting in cell death due to apoptosis or mitotic catastrophe. Thus, CpG hypermethylation-associated silencing of *BRCA1* causes hypersensitivity to PARP inhibitors, as also shown in *BRCA1*-deficient tumors due to genetic alterations.^{17,20} 53BP1, tumor protein P53 binding protein 1; *BRCA1*, *BRCA1* DNA repair associated; *BRCA2*, *BRCA2* DNA repair associated; PalB2, partner and localizer of *BRCA2*; Rad51, RAD51 recombinase; wt, wild-type.

DNA from tumors has led to the use of these alterations as potential biomarkers in early detection and diagnosis, as well as prognosis, in breast cancer. Panels of tumor-specific methylated genes in cell-free DNA with potential value for early detection of breast cancer have been described by Kloten et al³⁴ (*ITIH5*, *DKK3*, and *RASSF1A*) and Shan et al³⁵ (*SFN*, *CDKN2A*, *MLH1*, *HOXD13*, *PCDHGB7*, and *RASSF1A*). *RASSF1A* and *PITX2* hypermethylation has been associated with poor prognosis and pathogenesis in the early stages of breast cancer.^{36,37} Moreover, the role of *PITX2* hypermethylation in outcome prediction has been demonstrated in tamoxifen-treated, node-negative breast cancer, as well as in nontreated, HR⁺ cancer.^{38,39}

Epigenetic regulation is also a well-recognized mechanism controlling epithelial mesenchymal transition in breast cancer progression. Thus, hypermethylation of *CDH1* is a common event in breast tumors and has been associated with the triple-negative phenotype.^{40,41} *CDH1* hypermethylation is a poor-prognosis factor and has been postulated as a novel therapeutic target for the treatment of ER-negative or HER2-negative breast cancer with aggressive tumor biology.^{42,43} Furthermore, we have reported aberrant methylation of other epithelial mesenchymal transition–related genes in breast tumors, including *ITGA5*, *TINAGL1*, *FKBP10*, and *ESYT3*, with hypermethylation of the latter being a poor-prognosis factor in breast cancer.⁴⁴ Some remarkable epigenetically regulated genes that have been associated with breast carcinogenesis are listed in Table 1.

Genome-Wide Approaches in Epigenomic Research

In recent years, extraordinary advances in technology have revolutionized not only cancer genetics but also epigenetics. Genome-wide analyses of epigenetic marks, including DNA

methylation and histone modifications, are expanding our understanding about the disease and are providing new tools for cancer diagnosis, prognosis, and therapy. A list of technologies used in breast cancer epigenomic research discussed in this section is provided in Table 2.

A key advancement in the analysis of DNA methylation resulted from the demonstration that the treatment of DNA with sodium bisulfite deaminates the unmethylated cytosines, converting them to uracil while leaving 5-methylcytosine intact. Thus, DNA bisulfite treatment followed by the application of high-throughput technologies has allowed the generation of global DNA-methylation profiles. Next-generation sequencing technology has enabled genome-wide analysis of 5-methylcytosine nucleotides at single-nucleotide resolution by genome sequencing of bisulfite-treated DNA (whole-genome bisulfite sequencing). However, the high costs associated with sequencing continue to limit its application in large-scale patient cohorts. To decrease the cost of whole-genome bisulfite sequencing, reduced-representation bisulfite sequencing⁶⁶ technology was developed for sequencing a smaller, representative sample of the whole genome. Using a methylation-insensitive CpG restriction endonuclease (typically *MspI*) to generate fragments with CpG enrichment at the ends, this approach captures 85% of CpG islands and 60% of promoters.⁶⁷ Another option to decrease costs by sequencing a limited part of the DNA methylome is to enrich for those DNA fragments that are putatively methylated. This methodology is based on using antibodies directly against methylated DNA⁶⁸ or against methyl-CpG–binding domain proteins, which have a high affinity for binding to methylated cytosines.⁶⁹ The immunoprecipitated DNA can then be sequenced (methylated immunoprecipitated sequencing or methyl-CpG-binding domain proteins sequencing).⁷⁰ However, when a large number of samples need to be analyzed, bisulfite conversion–dependent methylation arrays are now

Table 1 Epigenetic Regulated Genes in Breast Cancer*

Gene	Biological and clinical relevance	References
<i>ESR1</i>	Carcinogenesis, associated with ER [−] breast tumors and PR status; poor prognosis and poor response to antiestrogen agents	13–15
<i>RASSF1A</i>	Carcinogenesis, mainly in HR ⁺ breast tumors; early detection of BC in cfDNA; poor prognosis in early stages of BC	13,16,18,25,27–30,34–37
<i>TIMP3</i>	Carcinogenesis, associated with ER [−] breast tumors	13,32
<i>BRCA1</i>	Carcinogenesis in TNBC, sensitivity to PARPi and DNA-damaging agents	19,21,25,32
<i>TWIST</i>	Carcinogenesis	25,27,34
<i>APC</i>	Carcinogenesis	25,28,32
<i>CDH13</i>	Carcinogenesis, associated with TNBC phenotype	25,29,31
<i>GSTP1</i>	Carcinogenesis, associated with lymph node–positive BC	25,30,32
<i>CCND2</i>	Carcinogenesis, mainly in ER ⁺ tumors	27,28,30,34
<i>HIN1</i>	Carcinogenesis, associated with HR ⁺ breast tumors	28,29,34
<i>CST6</i>	Carcinogenesis, poor prognosis in cfDNA in early stages of BC	32,33,40
<i>CDKN2A</i> (p16)	Carcinogenesis, early detection of BC in cfDNA	32,35,45
<i>PITX2</i>	Carcinogenesis, poor prognosis in HR ⁺ lymph node-negative BC	36–39
<i>CDH1</i>	Carcinogenesis, associated with TNBC phenotype	40–43

*In all of the listed genes, aberrant 5'-C-phosphate-G-3' (CpG) hypermethylation is the epigenetic event occurring in breast cancer.
+, positive; −, negative; BC, breast cancer; cfDNA, cell-free DNA; ER⁺, estrogen receptor–positive; ER[−], estrogen receptor–negative; HR⁺, hormone receptor–positive; PARPi, poly(ADP-ribose) polymerase inhibitor; PR, progesterone receptor; TNBC, triple-negative breast cancer.

Table 2 Genome-Wide Technologies and Their Use in Breast Cancer Epigenomic Research

Technology	Description	Application in breast cancer settings
GoldenGate Methylation Cancer Panel I ^{46*}	DNA methylation array (~1500 CpGs)	DNA methylation profiling and BC classification ^{47,48}
HumanMethylation27 BeadChip ^{49*}	DNA methylation array (~27,000 CpGs)	DNA methylation profiling and BC metastatic behavior ⁵⁰
HumanMethylation450 BeadChip ^{51,52*}	DNA methylation array (~450,000 CpGs)	DNA methylation profiling and BC stratification and prognosis ^{53–58}
Infinium MethylationEPIC BeadChip ^{59*} WGBS	DNA methylation array (~850,000 CpGs) Next-generation sequencing	DNA methylation profiling (ongoing) DNA methylation profiling and BC classification ^{57,58}
MBD-seq	Immunoprecipitation using antibodies against MBDs followed by next-generation sequencing of methylated DNA	DNA methylation profiling and BC stratification and prognosis ^{60,61}
ChIP-seq	ChIP using antibodies against histone modifications followed by next-generation sequencing	Histone modification profiles in BC subtypes ^{62,63} and prognosis ^{64,65}

*Illumina, San Diego, CA.

BC, breast cancer; ChIP, chromatin immunoprecipitation; CpG, 5'-C-phosphate-G-3'; MBD, methyl-CpG-binding domain protein; seq, sequencing; WGBS, whole-genome bisulfite sequencing.

the preferred alternative for assessing DNA methylation. The Infinium arrays (Illumina, San Diego, CA) have rapidly evolved, from the GoldenGate Methylation Cancer Panel I analyzing 1536 CpG dinucleotides,⁴⁶ the HumanMethylation27 BeadChip (approximately 27K CpGs),⁴⁹ and the HumanMethylation450 BeadChip (approximately 450K CpGs),^{51,52} to the newest version, the Infinium MethylationEPIC BeadChip (approximately 850K CpGs),⁵⁹ offering the most convenient platforms for genome-wide methylation profiling. The array interrogates >450,000 CpGs, including not only CpG islands, shores, and shelf regions surrounding the transcription start sites for coding genes, but also gene bodies and 3'-untranslated regions, in addition to intergenic regions derived from genome-wide association studies.⁵¹ The use of the Illumina 450K arrays has been broadly extended, potentiated by their use as the platform selected for The Cancer Genome Atlas (TCGA) studies⁷¹ and by their versatility in determining DNA-methylation patterns from formalin-fixed, paraffin-embedded samples.⁷² The new 850K platform (853,307 CpGs), incorporating CpG sites located in enhancer regions identified by the Encyclopedia of DNA Elements and Functional Annotation of the Mammalian Genome 5 projects, is becoming an increasingly valuable tool in massive epigenetic studies.⁵⁹

In addition to DNA methylation, high-throughput technologies are extensively used for studying histone modifications. Chromatin immunoprecipitation (ChIP) using antibodies against histone marks, followed by genomic sequencing (ChIP-seq), has become an indispensable tool for assessing epigenetic modifications.⁷³ Histone modifications are crucial regulators of gene expression, activating and silencing transcription by controlling the accessibility of DNA and by serving as binding substrates to recruit or exclude protein complexes.⁶ In general, acetylation, methylation, phosphorylation, and ubiquitination have been implicated in activation, whereas methylation, ubiquitination,

SUMOylation, deamination, and proline isomerization have been implicated in repression. However, it is context dependent; thus, methylation at H3K36 has a positive effect when on the coding region and a negative effect when in the promoter.⁶ Histone modifications can dictate the higher-order chromatin structure in which DNA is packaged and consequently can orchestrate the ordered recruitment of enzyme complexes to manipulate DNA. ChIP-seq technology has allowed the determination of the genome-wide distribution of histone marks that, integrated with transcriptome data from RNAseq approaches, have provided crucial clues about their role in transcriptional regulation. In the era of next-generation omics, formidable efforts to integrate genomics, epigenomics, transcriptomics, and proteomics data have been made. Consortia and projects involving institutions worldwide have been established. In the cancer field, a collaboration between the National Cancer Institute and the National Human Genome Research Institute set up TCGA in 2006. TCGA has generated multidimensional maps of the key genome changes in 33 types of cancer from >11,000 patients.⁷¹ TCGA data are publicly available and have become an extraordinary resource for the research community. The TCGA portal presently has >1000 samples for breast cancer, and integrated molecular analyses have already been published.⁵³ Additional compilations of omics data are available from the TNBC Database⁷⁴ and the International Cancer Genome Consortium (<http://www.icgc.org>, last accessed on August 10, 2017) with eight different projects in breast cancer.

Breast Cancer Epigenomics: Advances in DNA-Methylation Profiling

Genome-wide data generated in recent years have been crucial in unraveling the complexity and heterogeneous nature of breast cancer. First, findings from transcriptome

studies have shown that breast tumors can be classified into at least five molecular subtypes based on gene-expression profiles.^{75,76} Next, findings from studies using the GoldenGate DNA methylation array–based platform revealed that those molecular subtypes, especially basal-like, luminal A, and luminal B tumors, but also HER2-overexpressing tumors harbor specific methylation profiles,^{47,48} highlighting the relevance of epigenetic mechanisms in breast tumorigenesis and validating the use of DNA-methylation profiles as biomarkers for prognostic and therapeutic stratification of breast cancer patients. Using the following Infinium 27K platform, methylomes of breast cancers with diverse metastatic behavior were characterized, and the presence of a breast CpG island methylator phenotype was shown.⁵⁰ The breast CpG island methylator phenotype, characterized by coordinate hypermethylation at a large number of genes, was associated with low risks for metastasis and death, whereas the absence of that phenotype was associated with high risks for metastasis and death. Interestingly, the epigenomic alterations associated with the breast CpG island methylator phenotype were linked with many of the gene-expression differences observed in breast cancer–prognostic signatures such as MammaPrint (Agendia, Irvine, CA), indicating a fundamental epigenomic contribution to metastasis.⁵⁰ Using a more comprehensive technology, the Infinium 450K methylation array, the TCGA consortium has generated DNA-methylation profiles for >1000 breast tumor samples. Combining data from different platforms (DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays and sequencing, microRNA sequencing, and reverse-phase protein arrays), TCGA published a comprehensive molecular landscape of breast tumors.⁵³ Integrative analyses demonstrated the existence of four main breast tumor subtypes caused by different subsets of genetic and epigenetic abnormalities.⁵³

Using the same 450K methylation platform, our group identified two distinctive DNA-methylation signatures in breast cancer, validated also in the TCGA cohort.⁵⁴ These signatures underlie the definitions of two novel DNA methylation–based subtypes, referred to as Epi-Basal and Epi-LumB, associated with the biologically distinct and aggressive breast cancer subtypes basal-like and luminal-B, respectively. Importantly, that study identified a selected set of proxy markers for each of the two distinct signatures that are sufficient for classifying tumors according to DNA methylation–based subtypes, being a cost-effective method with potential clinical application.⁵⁴

Holm et al⁵⁵ also defined DNA-methylation epigenetic subgroups (epitypes) by studying whole-genome DNA-methylation profiles from 188 human breast tumors using 450K arrays. They identified seven epitypes that were validated in TCGA samples. By integrating analyses across multiple platforms, they demonstrated that the epitypes are associated with specific gene-expression subtypes, mutations, and DNA copy number aberrations. Interestingly,

epitypes were also associated with clinicopathologic features and patient outcomes. Furthermore, they found that DNA hypermethylation in luminal and basal-like tumors occurs in different chromatin contexts with different underlying regulatory potential in stem and mammary epithelial cells.⁵⁵

To identify epigenetic driver events in ER⁺ breast cancers, large-scale integrative analyses of DNA methylation and gene expression TCGA data have also been performed and validated in independent cohorts.⁵⁶ Using this strategy, Gao et al⁵⁶ identified epigenetic silencing of WNT signaling antagonists and bone morphogenetic proteins as key events underlying both luminal subtypes but especially of luminal-B breast cancer.

Technologies based on next-generation sequencing have also been applied to breast cancer research. DNA-methylation profiles generated by methyl-CpG–binding domain protein sequencing defined the first potential prognostic methylation signature of survival, specific for TNBCs.⁶¹ TNBCs are generally associated with a high risk for disease recurrence and shorter overall survival compared with non-TNBCs. However, it is still a heterogeneous group of cancers with varying prognoses, so tools for patient stratification into high- and low-risk subgroups are required. Using the methyl-CpG–binding domain protein sequencing approach, Stirzaker et al⁶¹ identified novel regions of differential methylation, and validated specific regions for TNBC using TCGA methylation data as an independent cohort. Based on the differentially methylated regions, they also identified three distinct methylation clusters of high-, medium-, or low-risk disease outcome. Moreover, they identified 17 individual differentially methylated regions for stratifying TNBC patients into good- and poor-prognosis groups.

Next-generation sequencing technologies, such as methyl-CpG–binding domain protein sequencing, have also been used for determining the aberrant methylation patterns of noncoding RNAs.⁶⁰ In the same way as in protein-coding genes, epigenetic alterations in noncoding RNAs can be used as biomarkers that discriminate between breast cancer phenotypes.⁶⁰

Breast Cancer Epigenomics: Patterns of Histone Marks

In addition to DNA methylation, high-throughput strategies have also transformed the way histone modifications are assessed. The contribution of ChIP-seq technology to epigenome mapping has been colossal. As commented at the beginning of this review, histone modifications are involved in the fine-tuning of gene expression. In addition to histone marks clearly associated with gene activation [eg, H3K4 trimethylation (me3)] or repression (eg, H3K27me3),⁵ from the first comprehensive genome-wide maps generated by ChIP-seq in 2007,^{77,78} an increasing

number of studies have revealed novel functions for histone modifications, highlighting the importance of combinatorial patterns of modifications in the accurate regulation of gene expression. In breast cancer research, histone-modification profiles, including mapping of *H3K4* acetylation and *H3K4* trimethylation, *H3K9* acetylation, and *H3K27* methylation, have been used for defining breast cancer subtypes and have been recognized as crucial players in tumorigenesis.^{62,63} For instance, *H3K4* acetylation enrichment has been correlated with breast cancer progression, estrogen response, and the epithelial mesenchymal transition oncogenic pathway, indicating it as a potential predictor of deregulated cancer-related pathways and a strong indicator of progression from initial transformation to aggressive metastatic phenotypes.⁶² In addition to the specific role of *H3K4* acetylation as an epigenetic hallmark of cellular transformation in breast cancer, these findings provide a compelling rationale for *H3K4* acetylation as a therapeutic target for breast cancer intervention. Histone lysine acetyltransferase inhibitors are presently in various stages of clinical trials and may prevent cancer progression from the earliest stages.⁶²

Genome-wide ChIP-seq analyses not only have demonstrated the dynamism of epigenomic mechanisms during breast cancer transformation, but also have defined specific events occurring during the course of the disease.⁶⁴ Using a cell-based transformation model stably expressing three oncogenes in primary breast epithelial cells, Zhao et al⁶⁴ reported reductions in histones H3K9me2 and H3K9me3, and elevations in the demethylases for H3K9me1 and H3K9me2 (KDM3A, or JMJD1A) as important events in breast cancer. The findings were corroborated in breast cancer patients, in whom KDM3A seems to regulate the tumorigenesis of breast cancer through down-regulating H3K9me2 on the *MYC* (alias *c-Myc*) and *PAX3* oncogenes.

Chromatin signatures unique to each breast cancer subtype (luminal, basal-like, and HER2) have also been identified by genome-wide binding patterns of H3K4me3 and H3K27me3 in breast cancer cell lines compared with mammary epithelial cells. Remarkably, subtype classifications based on histone modifications were significantly correlated with relapse-free survival outcomes in patients.⁶⁵

Noninvasive Epigenetic Biomarkers and Clinical Applications

Alterations in epigenetic modifications, hallmarks of tumor cells, are also a valuable tool in clinical settings. Applications are extended during the full course of the disease, from early-diagnosis biomarkers to therapeutic targets or markers of response to treatment.

A compilation of epigenetic biomarkers of breast cancer risk was recently reviewed by Terry et al.⁷⁹ The utility of DNA-methylation changes as potent biomarkers is supported by several facts, including that aberrant DNA

methylation is a common and early event in breast tumorigenesis. Moreover, DNA methylation is a stable modification and low amounts of samples are required for epigenetic analysis. An additional advantage is that DNA-methylation changes occurring during carcinogenesis can be detected in biological fluids. Although in cancer, tissue samples remain the gold standard resource for identifying biomarkers with potential clinical relevance, the value of liquid biopsies is increasingly recognized, based on the detection of DNA methylation in cell-free DNA from tumor or circulating tumor cells. The main advantage of liquid biopsies is its utility in monitoring disease progression and response to treatment in a noninvasive manner.^{33–35,80,81}

In breast cancer, findings from several studies have shown the value of DNA-methylation analyses of aspirated nipple fluid, which contains breast epithelial cells, providing a direct noninvasive sampling of fluid from the mammary ductal system.^{82–84}

Whole-genome bisulfite sequencing and genome-wide methylation from the 450K methylation arrays in peripheral blood from subjects who later developed breast cancer, compared with that from healthy subjects, have revealed DNA hypomethylation events occurring before diagnosis that may have potential utility as markers of breast cancer risk and in early detection.^{57,58} Furthermore, results from the Translational Breast Cancer Research Consortium 005 prospective study,⁸⁵ the first biomarker study designed specifically to prospectively test as a primary end point the role of DNA methylation in blood, were recently published, revealing the methylation of a panel of genes as a strong predictor of survival outcomes in metastatic breast cancer. Clinical application of these findings in risk stratification and disease monitoring is warranted.

Genome-wide studies have also been useful for identifying single genes that can be used as biomarkers for diagnosis or prognosis. Locus-specific identification of the DNA-methylation level by sequencing-based technologies such as direct bisulfite and pyrosequencing as well as alternatives such as methylation-specific PCR-based and matrix-assisted laser desorption/ionization–mass-spectrometry–based analysis are cost-effective strategies suitable for reliably assessing DNA-methylation levels.^{86–88}

Epigenetic-Based Therapies in Breast Cancer

Another emerging field is the development and clinical use of epigenetic drugs. By exploiting the leading role of epigenetics in tumor development and the reversibility of epigenetic modifications, promising novel epigenetics-based therapies are being developed.^{89,90} Several classes of drugs that target epigenetic mechanisms are in preclinical or clinical trials, and some of them have been approved by the US Food and Drug Administration for the treatment of some tumor types. Successful examples are the DNA-demethylating agents 5-azacytidine (Vidaza; Celgene,

Summit, NJ) and 5-aza-2'-deoxycytidine (decitabine) in the treatment of myelodysplastic syndrome and acute myeloid leukemia.^{91,92} These drugs inhibit DNA methylation globally, exerting pleiotropic effects on both abnormally silenced tumor suppressor genes frequently hypermethylated and associated with poor outcomes in hematologic tumors as *CDKN2B/p15*, but also other genes and repetitive DNAs, as shown in samples obtained from patients treated with these drugs.^{93–95} Despite the well-recognized success of epigenetic drugs in the treatment of myelodysplastic syndrome and acute myeloid leukemia, the benefits in solid tumors are limited. Conversely, a brighter future is expected for drugs targeting histone modifiers. Recent reports point to the use of bromodomain and extra terminal domain (BET) inhibitors.^{96–98} BET proteins act as epigenetic readers that directly bind to acetylated lysine on histone tails and other nuclear proteins, serving as a molecular scaffold in establishing RNA polymerase II specificity to regulate gene expression, including key oncogenes and antiapoptotic genes.⁹⁹ The most studied BET inhibitor is JQ1, which specifically targets the BET protein BRD4. JQ1 competitively binds to BET protein bromodomains, preventing interaction with acetylated histones, and exerts broad-spectrum antitumor activity, in part by selective inhibition of oncogenes, including *MYC*.^{100,101} Strong evidence has shown that JQ1 has potent activity in nonclinical models of several hematologic malignancies,^{102,103} and in classic *MYC*-driven tumors such as neuroblastoma,¹⁰⁴ as well as pediatric sarcoma¹⁰⁵ and pancreatic cancer¹⁰⁶ models. Of greater relevance, early clinical trials have provided evidence that the inhibition of BET proteins is effective against some human cancers, including NUT carcinoma, multiple myeloma, and acute myelogenous leukemia.^{107–110} With regard to breast cancer, our group recently showed that JQ1 exerts growth-inhibitory activity in human luminal breast cancer cell lines associated with the depletion of *MYC*. Moreover, the use of JQ1 in a luminal-B transgenic mouse model, MMTV-PyMT, delays the onset of the disease and increases survival.¹¹¹ Additionally, another study demonstrated a strong, long-lasting effect of combination therapy of JQ1 with the ER degrader fulvestrant in a tamoxifen-resistant breast cancer xenograft mouse model.⁹⁶ Thus, the role of JQ1 as a potent suppressor of the classic ER α signaling pathway warranted further study in tamoxifen-resistant settings. Strategies to target BET proteins are presently an expanding field, and vast efforts to develop enhanced BET protein degraders have been made. Strikingly, a recent study reported the development of a second-generation BET protein degrader, BETd-246, which exhibits superior selectivity, potency, and antitumor activity in TNBC xenograft models.¹¹²

HDAC is also an attractive target for therapy for breast cancer. Some HDAC inhibitors modify chromatin structure, leading to the restoration of expression of aberrantly silenced genes, which in turn is associated with growth inhibition and apoptosis in cancer cells. Clinical trials of

HDAC inhibitors have shown antitumor activity in several tumor types. For example, suberoylanilide hydroxamic acid/vorinostat and romidepsin have been approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma¹¹³ and peripheral T-cell lymphoma,¹¹⁴ respectively. Many attempts to use HDAC inhibitors in breast cancer treatment have been made, with studies showing potent response when HDAC inhibitors were combined with cytotoxic drugs, aromatase inhibitors, prodrugs, and ionizing radiation. Completed and ongoing clinical trials of HDAC inhibitors in breast cancer have been recently summarized in excellent reviews.^{115,116}

Overall, epigenomic studies have significantly enhanced our understanding of breast cancer heterogeneity and improved the characterization of the disease. High-throughput technologies, including genome-wide array-based platforms and strategies associated with next-generation sequencing, have dramatically expanded the identification of epigenetically regulated genes in breast cancer and all this information is being used for the benefit of patients. Better knowledge about the biological mechanisms involved in breast cancer initiation, progression, and metastasis is translating into clinical applications for diagnosis, prognosis, and therapy. The available technologies at this time promise an outstanding future for epigenomics and its contributions to medical practice.

Acknowledgments

A memorial tribute to our colleague and friend Dr. Jurgen Veeck, who made important contributions to breast cancer epigenetics research. Dr. Tomas Moran helped with figure preparation.

References

1. Waddington CH: The epigenotype. *Endeavour* 1942, 1:18–20.
2. Holliday R: The inheritance of epigenetic defects. *Science* 1987, 238:163–170.
3. Antequera F: Structure, function and evolution of CpG island promoters. *Cell Mol Life Sci* 2003, 60:1647–1658.
4. Esteller M: Epigenetics in cancer. *N Engl J Med* 2008, 358:1148–1159.
5. Tessarz P, Kouzarides T: Histone core modifications regulating nucleosome structure and dynamics. *Nat Rev Mol Cell Biol* 2014, 15:703–708.
6. Kouzarides T: Chromatin modifications and their function. *Cell* 2007, 128:693–705.
7. Chi P, Allis CD, Wang GG: Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer* 2010, 10:457–469.
8. Feinberg AP, Vogelstein B: Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983, 301:89–92.
9. Valencia OM, Samuel SE, Viscusi RK, Riall TS, Neumayer LA, Aziz H: The role of genetic testing in patients with breast cancer: a review. *JAMA Surg* 2017, 152:589–594.
10. Pourteimoor V, Mohammadi-Yeganeh S, Paryan M: Breast cancer classification and prognostication through diverse systems along with

- recent emerging findings in this respect; the dawn of new perspectives in the clinical applications. *Tumour Biol* 2016, 37:14479–14499
11. Sonnenblick A, Piccart M: Adjuvant systemic therapy in breast cancer: quo vadis? *Ann Oncol* 2015, 26:1629–1634
 12. Giacinti L, Claudio PP, Lopez M, Giordano A: Epigenetic information and estrogen receptor alpha expression in breast cancer. *Oncologist* 2006, 11:1–8
 13. Widschwendter M, Siegmund KD, Muller HM, Fiegl H, Marth C, Muller-Holzner E, Jones PA, Laird PW: Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res* 2004, 64:3807–3813
 14. Gaudet MM, Campan M, Figueroa JD, Yang XR, Lissowska J, Peplonska B, Brinton LA, Rimm DL, Laird PW, Garcia-Closas M, Sherman ME: DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol Biomarkers Prev* 2009, 18:3036–3043
 15. Martínez-Galán J, Torres-Torres B, Nunez MI, Lopez-Penalver J, Del Moral R, Ruiz De Almodovar JM, Menjon S, Concha A, Chamorro C, Rios S, Delgado JR: ESR1 gene promoter region methylation in free circulating DNA and its correlation with estrogen receptor protein expression in tumor tissue in breast cancer patients. *BMC Cancer* 2014, 14:59
 16. Kajabova V, Smolkova B, Zmetakova I, Sebova K, Krivulcik T, Bella V, Kajo K, Machalekova K, Fridrichova I: RASSF1A promoter Methylation levels positively correlate with estrogen receptor expression in breast cancer patients. *Transl Oncol* 2013, 6: 297–304
 17. Li ML, Greenberg RA: Links between genome integrity and BRCA1 tumor suppression. *Trends Biochem Sci* 2012, 37:418–424
 18. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB, Herman JG: Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 2000, 92:564–569
 19. Veeck J, Roperio S, Setien F, Gonzalez-Suarez E, Osorio A, Benitez J, Herman JG, Esteller M: BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. *J Clin Oncol* 2010, 28:e563–e564; author reply e565–e566
 20. Lord CJ, Ashworth A: Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat Med* 2013, 19:1381–1388
 21. Stefansson OA, Villanueva A, Vidal A, Marti L, Esteller M: BRCA1 epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer. *Epigenetics* 2012, 7: 1225–1229
 22. Ter Brugge P, Kristel P, van der Burg E, Boon U, de Maaker M, Lips E, Mulder L, de Ruiter J, Moutinho C, Gevensleben H, Marangoni E, Majewski I, Jozwiak K, Kloosterman W, van Roosmalen M, Duran K, Hogervorst F, Turner N, Esteller M, Cuppen E, Wesseling J, Jonkers J: Mechanisms of therapy resistance in patient-derived xenograft models of BRCA1-deficient breast cancer. *J Natl Cancer Inst* 2016, 108
 23. Yamaguchi T, Mukai H, Yamashita S, Fujii S, Ushijima T: Comprehensive DNA methylation and extensive mutation analyses of HER2-positive breast cancer. *Oncology* 2015, 88:377–384
 24. Veeck J, Wild PJ, Fuchs T, Schuffler PJ, Hartmann A, Knuchel R, Dahl E: Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. *BMC Cancer* 2009, 9:217
 25. Huang KT, Mikeska T, Li J, Takano EA, Millar EK, Graham PH, Boyle SE, Campbell IG, Speed TP, Dobrovic A, Fox SB: Assessment of DNA methylation profiling and copy number variation as indications of clonal relationship in ipsilateral and contralateral breast cancers to distinguish recurrent breast cancer from a second primary tumour. *BMC Cancer* 2015, 15:669
 26. Ai L, Tao Q, Zhong S, Fields CR, Kim WJ, Lee MW, Cui Y, Brown KD, Robertson KD: Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* 2006, 27:1341–1348
 27. Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapides A, Garrett E, Argani P, Sukumar S: Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res* 2004, 64:4442–4452
 28. Jeronimo C, Monteiro P, Henrique R, Dinis-Ribeiro M, Costa I, Costa VL, Filipe L, Carvalho AL, Hoque MO, Pais I, Leal C, Teixeira MR, Sidransky D: Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions. *Breast Cancer Res Treat* 2008, 109:27–34
 29. Feng W, Shen L, Wen S, Rosen DG, Jelinek J, Hu X, Huan S, Huang M, Liu J, Sahin AA, Hunt KK, Bast RC Jr, Shen Y, Issa JP, Yu Y: Correlation between CpG methylation profiles and hormone receptor status in breast cancers. *Breast Cancer Res* 2007, 9:R57
 30. Sunami E, Shinozaki M, Sim MS, Nguyen SL, Vu AT, Giuliano AE, Hoon DS: Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. *Breast Cancer Res* 2008, 10:R46
 31. Toyooka KO, Toyooka S, Virmani AK, Sathyanarayana UG, Euhus DM, Gilcrease M, Minna JD, Gazdar AF: Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. *Cancer Res* 2001, 61:4556–4560
 32. Radpour R, Kohler C, Haghighi MM, Fan AX, Holzgreve W, Zhong XY: Methylation profiles of 22 candidate genes in breast cancer using high-throughput MALDI-TOF mass array. *Oncogene* 2009, 28:2969–2978
 33. Chimonidou M, Tzitzira A, Strati A, Sotiropoulou G, Sfikas C, Malamos N, Georgoulas V, Lianidou E: CST6 promoter methylation in circulating cell-free DNA of breast cancer patients. *Clin Biochem* 2013, 46:235–240
 34. Kloten V, Becker B, Winner K, Schrauder MG, Fasching PA, Anzener T, Veeck J, Hartmann A, Knuchel R, Dahl E: Promoter hypermethylation of the tumor-suppressor genes ITIH5, DKK3, and RASSF1A as novel biomarkers for blood-based breast cancer screening. *Breast Cancer Res* 2013, 15:R4
 35. Shan M, Yin H, Li J, Li X, Wang D, Su Y, Niu M, Zhong Z, Wang J, Zhang X, Kang W, Pang D: Detection of aberrant methylation of a six-gene panel in serum DNA for diagnosis of breast cancer. *Oncotarget* 2016, 7:18485–18494
 36. Gobel G, Auer D, Gaugg I, Schneitter A, Lesche R, Muller-Holzner E, Marth C, Daxenbichler G: Prognostic significance of methylated RASSF1A and PITX2 genes in blood- and bone marrow plasma of breast cancer patients. *Breast Cancer Res Treat* 2011, 130: 109–117
 37. Jezkova E, Kajo K, Zubor P, Grendar M, Malicherova B, Mendelova A, Dokus K, Lasabova Z, Plank L, Danko J: Methylation in promoter regions of PITX2 and RASSF1A genes in association with clinicopathological features in breast cancer patients. *Tumour Biol* 2016, 37:15707–15719
 38. Nimmrich I, Sieuwerts AM, Meijer-van Gelder ME, Schwöpe I, Bolt-de Vries J, Harbeck N, Koenig T, Hartmann O, Kluth A, Dietrich D, Magdolen V, Portengen H, Look MP, Klijn JG, Lesche R, Schmitt M, Maier S, Foekens JA, Martens JW: DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients. *Breast Cancer Res Treat* 2008, 111:429–437
 39. Harbeck N, Nimmrich I, Hartmann A, Ross JS, Cufer T, Grutzmann R, Kristiansen G, Paradiso A, Hartmann O, Margossian A, Martens J, Schwöpe I, Lukas A, Müller V, Milde-Langosch K, Nahrig J, Foekens J, Maier S, Schmitt M, Lesche R: Multicenter study using paraffin-embedded tumor tissue testing PITX2 DNA methylation as a marker for outcome prediction in tamoxifen-treated, node-negative breast cancer patients. *J Clin Oncol* 2008, 26:5036–5042

40. Roll JD, Rivenbark AG, Sandhu R, Parker JS, Jones WD, Carey LA, Livasy CA, Coleman WB: 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: 276–287
41. Naghitorabi M, Mohammadi-Asl J, Sadeghi HM, Rabbani M, Jafarian-Dehkordi A, Javanmard SH: Quantitation of CDH1 promoter methylation in formalin-fixed paraffin-embedded tissues of breast cancer patients using differential high resolution melting analysis. *Adv Biomed Res* 2016, 5:91
42. Liu J, Sun X, Qin S, Wang H, Du N, Li Y, Pang Y, Wang C, Xu C, Ren H: CDH1 promoter methylation correlates with decreased gene expression and poor prognosis in patients with breast cancer. *Oncol Lett* 2016, 11:2635–2643
43. Huang R, Ding P, Yang F: Clinicopathological significance and potential drug target of CDH1 in breast cancer: a meta-analysis and literature review. *Drug Des Devel Ther* 2015, 9:5277–5285
44. Carmona FJ, Davalos V, Vidal E, Gomez A, Heyn H, Hashimoto Y, Vizoso M, Martinez-Cardus A, Sayols S, Ferreira HJ, Sanchez-Mut JV, Moran S, Margeli M, Castella E, Berdasco M, Stefansson OA, Eyfjord JE, Gonzalez-Suarez E, Dopazo J, Orozco M, Gut IG, Esteller M: A comprehensive DNA methylation profile of epithelial-to-mesenchymal transition. *Cancer Res* 2014, 74: 5608–5619
45. Spitzwieser M, Entfellner E, Werner B, Pulverer W, Pfeiler G, Hacker S, Cichna-Markl M: Hypermethylation of CDKN2A exon 2 in tumor, tumor-adjacent and tumor-distant tissues from breast cancer patients. *BMC Cancer* 2017, 17:260
46. Bibikova M, Lin Z, Zhou L, Chudin E, Garcia EW, Wu B, Doucet D, Thomas NJ, Wang Y, Vollmer E, Goldmann T, Seifart C, Jiang W, Barker DL, Chee MS, Floros J, Fan JB: High-throughput DNA methylation profiling using universal bead arrays. *Genome Res* 2006, 16:383–393
47. Holm K, Hegardt C, Staaf J, Vallon-Christersson J, Jonsson G, Olsson H, Borg A, Ringner M: Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. *Breast Cancer Res* 2010, 12:R36
48. Bediaga NG, Acha-Sagredo A, Guerra I, Viguri A, Albaina C, Ruiz Diaz I, Rezola R, Alberdi MJ, Dopazo J, Montaner D, Renobales M, Fernandez AF, Field JK, Fraga MF, Liloglou T, de Pancorbo MM: DNA methylation epigenotypes in breast cancer molecular subtypes. *Breast Cancer Res* 2010, 12:R77
49. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, Gunderson KL: Genome-wide DNA methylation profiling using Infinium(R) assay. *Epigenomics* 2009, 1:177–200
50. Fang F, Turcan S, Rimner A, Kaufman A, Giri D, Morris LG, Shen R, Seshan V, Mo Q, Heguy A, Baylin SB, Ahuja N, Viale A, Massague J, Norton L, Vahdat LT, Moynahan ME, Chan TA: Breast cancer methylomes establish an epigenomic foundation for metastasis. *Sci Transl Med* 2011, 3:75ra25
51. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M: Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* 2011, 6: 692–702
52. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP, Gunderson KL, Fan JB, Shen R: High density DNA methylation array with single CpG site resolution. *Genomics* 2011, 98:288–295
53. Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. *Nature* 2012, 490:61–70
54. Stefansson OA, Moran S, Gomez A, Sayols S, Arribas-Jorba C, Sandoval J, Hilmarsdottir H, Olafsdottir E, Tryggvadottir L, Jonasson JG, Eyfjord J, Esteller M: A DNA methylation-based definition of biologically distinct breast cancer subtypes. *Mol Oncol* 2015, 9:555–568
55. Holm K, Staaf J, Lauss M, Aine M, Lindgren D, Bendahl PO, Vallon-Christersson J, Barkardottir RB, Hoglund M, Borg A, Jonsson G, Ringner M: An integrated genomics analysis of epigenetic subtypes in human breast tumors links DNA methylation patterns to chromatin states in normal mammary cells. *Breast Cancer Res* 2016, 18:27
56. Gao Y, Jones A, Fasching PA, Ruebner M, Beckmann MW, Widschwendter M, Teschendorff AE: The integrative epigenomic-transcriptomic landscape of ER positive breast cancer. *Clin Epigenetics* 2015, 7:126
57. van Veldhoven K, Polidoro S, Baglietto L, Severi G, Sacerdote C, Panico S, Mattiello A, Palli D, Masala G, Krogh V, Agnoli C, Tumino R, Frasca G, Flower K, Curry E, Orr N, Tomczyk K, Jones ME, Ashworth A, Swerdlow A, Chadeau-Hyam M, Lund E, Garcia-Closas M, Sandanger TM, Flanagan JM, Vineis P: Epigenome-wide association study reveals decreased average methylation levels years before breast cancer diagnosis. *Clin Epigenetics* 2015, 7:67
58. Severi G, Southey MC, English DR, Jung CH, Lonie A, McLean C, Tsimiklis H, Hopper JL, Giles GG, Baglietto L: Epigenome-wide methylation in DNA from peripheral blood as a marker of risk for breast cancer. *Breast Cancer Res Treat* 2014, 148:665–673
59. Moran S, Arribas C, Esteller M: Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics* 2016, 8:389–399
60. Li Y, Zhang Y, Li S, Lu J, Chen J, Wang Y, Li Y, Xu J, Li X: Genome-wide DNA methylome analysis reveals epigenetically dysregulated non-coding RNAs in human breast cancer. *Sci Rep* 2015, 5: 8790
61. Stirzaker C, Zotenko E, Song JZ, Qu W, Nair SS, Locke WJ, Stone A, Armstrong NJ, Robinson MD, Dobrovic A, Avery-Kiejda KA, Peters KM, French JD, Stein S, Korbic DJ, Trau M, Forbes JF, Scott RJ, Brown MA, Francis GD, Clark SJ: Methylome sequencing in triple-negative breast cancer reveals distinct methylation clusters with prognostic value. *Nat Commun* 2015, 6:5899
62. Messier TL, Gordon JA, Boyd JR, Tye CE, Browne G, Stein JL, Lian JB, Stein GS: Histone H3 lysine 4 acetylation and methylation dynamics define breast cancer subtypes. *Oncotarget* 2016, 7: 5094–5109
63. Judes G, Dagdemir A, Karsli-Ceppegolu S, Lebert A, Echegut M, Ngollo M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D: H3K4 acetylation, H3K9 acetylation and H3K27 methylation in breast tumor molecular subtypes. *Epigenomics* 2016, 8:909–924
64. Zhao QY, Lei PJ, Zhang X, Zheng JY, Wang HY, Zhao J, Li YM, Ye M, Li L, Wei G, Wu M: Global histone modification profiling reveals the epigenomic dynamics during malignant transformation in a four-stage breast cancer model. *Clin Epigenetics* 2016, 8:34
65. Chen X, Hu H, He L, Yu X, Liu X, Zhong R, Shu M: A novel subtype classification and risk of breast cancer by histone modification profiling. *Breast Cancer Res Treat* 2016, 157:267–279
66. Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R: Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res* 2005, 33:5868–5877
67. Gu H, Smith ZD, Bock C, Boyle P, Gnirke A, Meissner A: Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nat Protoc* 2011, 6: 468–481
68. Jacinto FV, Ballestar E, Esteller M: Methyl-DNA immunoprecipitation (MeDIP): hunting down the DNA methylome. *Biotechniques* 2008, 44:35,37,39passim
69. Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F, Esteller M: A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Res* 2006, 66:8342–8346
70. Li N, Ye M, Li Y, Yan Z, Butcher LM, Sun J, Han X, Chen Q, Zhang X, Wang J: Whole genome DNA methylation analysis based on high throughput sequencing technology. *Methods* 2010, 52: 203–212

71. Grossman RL, Heath AP, Ferretti V, Varmus HE, Lowy DR, Kibbe WA, Staudt LM: Toward a shared vision for cancer genomic data. *N Engl J Med* 2016, 375:1109–1112
72. Moran S, Vizoso M, Martinez-Cardus A, Gomez A, Matias-Guiu X, Chiavenna SM, Fernandez AG, Esteller M: Validation of DNA methylation profiling in formalin-fixed paraffin-embedded samples using the Infinium HumanMethylation450 Microarray. *Epigenetics* 2014, 9:829–833
73. Park PJ: ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet* 2009, 10:669–680
74. Raju R, Paul AM, Asokachandran V, George B, Radhamony L, Vinaykumar M, Girijadevi R, Pillai MR: The Triple-Negative Breast Cancer Database: an omics platform for reference, integration and analysis of triple-negative breast cancer data. *Breast Cancer Res* 2014, 16:490
75. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D: Molecular portraits of human breast tumours. *Nature* 2000, 406:747–752
76. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, Borresen-Dale AL: Gene expression patterns of breast carcinomas distinguishing tumor subtypes with clinical implications. *Proc Natl Acad Sci U S A* 2001, 98:10869–10874
77. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K: High-resolution profiling of histone methylations in the human genome. *Cell* 2007, 129:823–837
78. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE: Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 2007, 448:553–560
79. Terry MB, McDonald JA, Wu HC, Eng S, Santella RM: Epigenetic biomarkers of breast cancer risk: across the breast cancer prevention continuum. *Adv Exp Med Biol* 2016, 882:33–68
80. Wan JC, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N: Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017, 17:223–238
81. Siravegna G, Marsoni S, Siena S, Bardelli A: Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol* 2017, 14:531–548
82. de Groot JS, Moelans CB, Elias SG, Jo Fackler M, van Domselaar R, Suijkerbuijk KP, Witkamp AJ, Sukumar S, van Diest PJ, van der Wall E: DNA promoter hypermethylation in nipple fluid: a potential tool for early breast cancer detection. *Oncotarget* 2016, 7:24778–24791
83. Suijkerbuijk KP, van der Wall E, Vooijs M, van Diest PJ: Molecular analysis of nipple fluid for breast cancer screening. *Pathobiology* 2008, 75:149–152
84. Krassenstein R, Sauter E, Dulaimi E, Battagli C, Ehya H, Klein-Szanto A, Cairns P: Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. *Clin Cancer Res* 2004, 10:28–32
85. Visvanathan K, Fackler MS, Zhang Z, Lopez-Bujanda ZA, Jeter SC, Sokoll LJ, Garrett-Mayer E, Cope LM, Umbricht CB, Euhus DM, Forero A, Storniolo AM, Nanda R, Lin NU, Carey LA, Ingle JN, Sukumar S, Wolff AC: Monitoring of serum DNA methylation as an early independent marker of response and survival in metastatic breast cancer: TBCRC 005 prospective biomarker study. *J Clin Oncol* 2017, 35:751–758
86. Tost J, Schatz P, Schuster M, Berlin K, Gut IG: Analysis and accurate quantification of CpG methylation by MALDI mass spectrometry. *Nucleic Acids Res* 2003, 31:e50
87. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D: Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* 2005, 102:15785–15790
88. Heyn H, Esteller M: DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* 2012, 13:679–692
89. Simo-Riudalbas L, Esteller M: Targeting the histone orthography of cancer: drugs for writers, erasers and readers. *Br J Pharmacol* 2015, 172:2716–2732
90. Stefansson OA, Esteller M: Epigenetic modifications in breast cancer and their role in personalized medicine. *Am J Pathol* 2013, 183:1052–1063
91. Scott LJ: Azacitidine: a review in myelodysplastic syndromes and acute myeloid leukaemia. *Drugs* 2016, 76:889–900
92. Yun S, Vincelette ND, Abraham I, Robertson KD, Fernandez-Zapico ME, Patnaik MM: Targeting epigenetic pathways in acute myeloid leukemia and myelodysplastic syndrome: a systematic review of hypomethylating agents trials. *Clin Epigenetics* 2016, 8:68
93. Yang AS, Doshi KD, Choi SW, Mason JB, Mannari RK, Gharybian V, Luna R, Rashid A, Shen L, Estecio MR, Kantarjian HM, Garcia-Manero G, Issa JP: DNA methylation changes after 5-aza-2'-deoxycytidine therapy in patients with leukemia. *Cancer Res* 2006, 66:5495–5503
94. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z, Ferrajoli A, Wierda W, Shan J, Davis J, Giles F, Saba HI, Issa JP: Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood* 2007, 109:52–57
95. Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wijermans P, Jones PA, Lubbert M: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. *Blood* 2002, 100:2957–2964
96. Feng Q, Zhang Z, Shea MJ, Creighton CJ, Coarfa C, Hilsenbeck SG, Lanz R, He B, Wang L, Fu X, Nardone A, Song Y, Bradner J, Mitsiades N, Mitsiades CS, Osborne CK, Schiff R, O'Malley BW: An epigenomic approach to therapy for tamoxifen-resistant breast cancer. *Cell Res* 2014, 24:809–819
97. Nieto-Jimenez C, Alcaraz-Sanabria A, Perez-Pena J, Corrales-Sanchez V, Serrano-Heras G, Galan-Moya EM, Serrano-Oviedo L, Montero JC, Burgos M, Llopis J, Pandiella A, Ocana A: Targeting basal-like breast tumors with bromodomain and extraterminal domain (BET) and polo-like kinase inhibitors. *Oncotarget* 2017, 8:19478–19490
98. Wali VB, Langdon CG, Held MA, Platt JT, Patwardhan GA, Safonov A, Aktas B, Pusztai L, Stern DF, Hatzis C: Systematic drug screening identifies tractable targeted combination therapies in triple-negative breast cancer. *Cancer Res* 2017, 77:566–578
99. Filippakopoulos P, Knapp S: Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat Rev Drug Discov* 2014, 13:337–356
100. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastiris E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE, Mitsiades CS: BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011, 146:904–917
101. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, Bergeron L, Sims RJ 3rd: Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci U S A* 2011, 108:16669–16674
102. Chaidos A, Caputo V, Gouvedenou K, Liu B, Marigo I, Chaudhry MS, Rotolo A, Tough DF, Smithers NN, Bassil AK, Chapman TD, Harker NR, Barbash O, Tummino P, Al-Mahdi N, Haynes AC, Cutler L, Le B, Rahemtulla A, Roberts I, Kleijnen M, Witherington JJ, Parr NJ, Prinjha RK, Karadimitris A: Potent anti-melanoma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood* 2014, 123:697–705

103. Chaidos A, Caputo V, Karadimitris A: Inhibition of bromodomain and extra-terminal proteins (BET) as a potential therapeutic approach in haematological malignancies: emerging preclinical and clinical evidence. *Ther Adv Hematol* 2015, 6:128–141
104. Puissant A, Frumm SM, Alexe G, Bassil CF, Qi J, Chanthery YH, Nekritz EA, Zeid R, Gustafson WC, Greninger P, Garnett MJ, McDermott U, Benes CH, Kung AL, Weiss WA, Bradner JE, Stegmaier K: Targeting MYCN in neuroblastoma by BET bromodomain inhibition. *Cancer Discov* 2013, 3:308–323
105. Bid HK, Phelps DA, Xiao L, Guttridge DC, Lin J, London C, Baker LH, Mo X, Houghton PJ: The Bromodomain BET inhibitor JQ1 suppresses tumor angiogenesis in models of childhood sarcoma. *Mol Cancer Ther* 2016, 15:1018–1028
106. Yamamoto K, Tateishi K, Kudo Y, Hoshikawa M, Tanaka M, Nakatsuka T, Fujiwara H, Miyabayashi K, Takahashi R, Tanaka Y, Ijichi H, Nakai Y, Isayama H, Morishita Y, Aoki T, Sakamoto Y, Hasegawa K, Kokudo N, Fukayama M, Koike K: Stromal remodeling by the BET bromodomain inhibitor JQ1 suppresses the progression of human pancreatic cancer. *Oncotarget* 2016, 7:61469–61484
107. Odore E, Lokiec F, Cvitkovic E, Bekradda M, Herait P, Bourdel F, Kahatt C, Raffoux E, Stathis A, Thieblemont C, Quesnel B, Cunningham D, Riveiro ME, Rezai K: Phase I population pharmacokinetic assessment of the oral bromodomain inhibitor OTX015 in patients with haematologic malignancies. *Clin Pharmacokinet* 2016, 55:397–405
108. Berthon C, Raffoux E, Thomas X, Vey N, Gomez-Roca C, Yee K, Taussig DC, Rezai K, Roumier C, Herait P, Kahatt C, Quesnel B, Michallet M, Recher C, Lokiec F, Preudhomme C, Dombret H: Bromodomain inhibitor OTX015 in patients with acute leukaemia: a dose-escalation, phase 1 study. *Lancet Haematol* 2016, 3:e186–e195
109. Stathis A, Zucca E, Bekradda M, Gomez-Roca C, Delord JP, de La Motte Rouge T, Uro-Coste E, de Braud F, Pelosi G, French CA: Clinical response of carcinomas harboring the BRD4-NUT oncoprotein to the targeted bromodomain inhibitor OTX015/MK-8628. *Cancer Discov* 2016, 6:492–500
110. Amorim S, Stathis A, Gleeson M, Iyengar S, Magarotto V, Leleu X, Morschhauser F, Karlin L, Broussais F, Rezai K, Herait P, Kahatt C, Lokiec F, Salles G, Facon T, Palumbo A, Cunningham D, Zucca E, Thieblemont C: Bromodomain inhibitor OTX015 in patients with lymphoma or multiple myeloma: a dose-escalation, open-label, pharmacokinetic, phase 1 study. *Lancet Haematol* 2016, 3:e196–e204
111. Pérez-Salvia M, Simó-Riudalbas L, Llinàs-Arias P, Roa L, Setien F, Soler M, Castro de Moura M, Bradner JE, Gonzalez-Suarez E, Moutinho C, Esteller M: Bromodomain inhibition shows antitumoral activity in mice and human luminal breast cancer. *Oncotarget* 2017, 8:51621–51629
112. Bai L, Zhou B, Yang CY, Ji J, McEachern D, Przybranowski S, Jiang H, Hu J, Xu F, Zhao Y, Liu L, Fernandez-Salas E, Xu J, Dou Y, Wen B, Sun D, Meagher J, Stuckey J, Hayes DF, Li S, Ellis MJ, Wang S: Targeted degradation of BET proteins in triple-negative breast cancer. *Cancer Res* 2017, 77:2476–2487
113. Mann BS, Johnson JR, He K, Sridhara R, Abraham S, Booth BP, Verbois L, Morse DE, Jee JM, Pope S, Harapanhalli RS, Dagher R, Farrell A, Justice R, Pazdur R: Vorinostat for treatment of cutaneous manifestations of advanced primary cutaneous T-cell lymphoma. *Clin Cancer Res* 2007, 13:2318–2322
114. Barbarotta L, Hurley K: Romidepsin for the Treatment of Peripheral T-Cell Lymphoma. *J Adv Pract Oncol* 2015, 6:22–36
115. Damaskos C, Valsami S, Kontos M, Spartalis E, Kalampokas T, Kalampokas E, Athanasiou A, Moris D, Daskalopoulou A, Davakis S, Tsourouflis G, Kontzoglou K, Perrea D, Nikiteas N, Dimitroulis D: Histone deacetylase inhibitors: an attractive therapeutic strategy against breast cancer. *Anticancer Res* 2017, 37:35–46
116. Walsh L, Gallagher WM, O'Connor DP, Ní Chonghaile T: Diagnostic and therapeutic implications of histone epigenetic modulators in breast cancer. *Expert Rev Mol Diagn* 2016, 16:541–551