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REVIEW ARTICLE

The Epigenetics of Triple-Negative and Basal-Like Breast Cancer: Current Knowledge

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Breast cancer has the highest incidence among all malignancies diagnosed in women. Therapies have significantly improved over the years due to extensive molecular and clinical research; in a large number of cases, targeted therapies have provided better prognosis. However, one specific subtype remains elusive to targeted therapies—the triple-negative breast cancer. This immunohistochemically defined subtype is resistant to both endocrine and targeted therapies, leading to its poor prognosis. A field that is of great promise in current cancer research is epigenetics. By studying the epigenetic mechanisms underlying tumorigenesis—DNA methylation, histone modifications, and noncoding RNAs—advances in cancer treatment, diagnosis, and prevention are possible. This review aims to synthesize the epigenetic discoveries that have been made related to the triple-negative breast cancer.

Key Words: *Breast neoplasms, DNA methylation, Histone code, Triple negative breast neoplasms, Untranslated RNA*

INTRODUCTION

Breast cancer is the most diagnosed type of cancer in women, and it has the second highest mortality rate in this group, following only lung cancer [1]. Triple-negative breast cancer (TNBC) defines a subtype of breast cancer that does not express estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2). Approximately 20% of breast cancers in women are TNBC, and this type is more aggressive and often diagnosed in younger patients [2,3]. It is a clinical challenge to treat TNBC, as no therapeutic targets have so far been successfully described, and its prognosis remains modest.

Different research groups have proposed molecular classifications based on gene expression profiling (Figure 1). Thus, five intrinsic subtypes of breast cancer have initially been defined [4,5]. Subsequently, in order to translate these findings and make them suitable for clinical application, immunohistochemical equivalents to the intrinsic subtypes have been established [6,7]. Among these subtypes of TNBC, approximately 80% are basal-like [8,9]. However, not all basal-like breast cancers are triple-negative, and up to 20% of basal-like tumors are either ER+ or HER2+ [8]. Other subtypes include the claudin-low [10] and the interferon-rich, which is closely related to TNBC [11].

Most gene expression profiling studies on TNBC have been conducted in order to establish better prognostic or predictive factors. One of the first studies by Lehmann et al. in 2011 described six subtypes: two basal-like (BL1 and BL2), one immunomodulatory (IM), one mesenchymal (M), one mesenchymal stem-like (MSL), and one luminal androgen receptor (LAR) [12]. The MSL and IM subtypes were subsequently removed because they are formed by tumor-associated stromal cells and infiltrating lymphocytes, respectively [13] (Figure 1). Burstein et al. [14], in 2015, described four subtypes with clinical significance: luminal-AR (LAR), mesenchymal (MES), basal-like immune-suppressed (BLIS), and basal-like immune-activated (BLIA). Among these, BLIA and BLIS had the best and worst prognoses, respectively, according to both dis-

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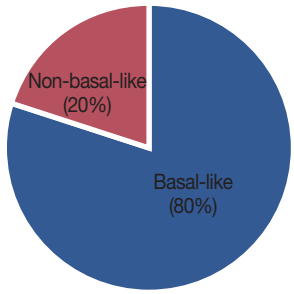
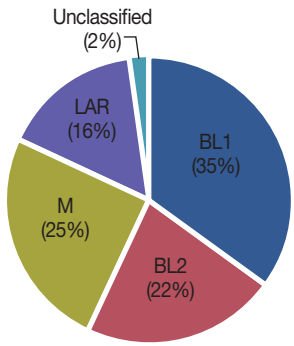


Intrinsic subtypes of breast cancer	
Luminal A	ER+, PR+, HER2–, Ki-67 low
Luminal B	ER+, PR+, HER2–, Ki-67 high ER+, PR+, HER2+, Ki-67 high
HER2-enriched	ER–, PR–, HER2+
Basal-like	ER–, PR–, HER2– Expression of keratins 5 and 17, laminin and fatty acid binding protein 7
Normal-like	ER+, PR+, HER2–, Ki-67 low Genes expressed by adipose tissue and other nonepithelial cell types

A



TNBCtype-4	
BL1	Enriched in cell division pathway components and DNA damage response (ATR/BRCA) pathways
BL2	Growth factor signaling (EGF pathway, NGF pathway, MET pathway, Wnt/β-catenin, and IGF1R pathway), glycolysis and gluconeogenesis and expression of myoepithelial markers (TP63 and MME)
M	Genes involved in motility, extracellular matrix, cell differentiation pathways and genes associated with epithelial to mesenchymal transition (EMT)
LAR	Enriched for genes involved in steroid synthesis and androgen/estrogen metabolism, including high levels of androgen receptor (AR) and its downstream targets and coactivators

B

Figure 1. Classification systems developed for triple-negative breast cancer (TNBC). (A) Intrinsic subtypes of breast cancer, first established in 2001 by Sørlie et al. [5]. This classification is most widely used by both clinicians and researchers due to numerous confirmed prognostic, predictive and therapeutic correlations. The pie chart represents the distribution of these intrinsic subtypes among the imunohistochemically defined TNBC, the majority being represented by basal-like tumors. (B) TNBCtype-4 comprised of six subtypes was established in 2011 by Lehmann et al. [12] and subsequently redefined to TNBCtype-4 in 2016 [13]. The pie chart represents the distribution of these subtypes among TNBC, again the majority being represented by basal-like tumors.
ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; BL = basal-like; M = mesenchymal; LAR = luminal androgen receptor; EGF = epidermal growth factor; NGF = nerve growth factor; MET = tyrosine-protein kinase Met encoded by the *MET* gene; IGF1R = insulin-like growth factor 1 receptor.

ease-free and disease-specific survival parameters. Moreover, subtype-specific targets were identified [14]. Another study identified three clusters of TNBC by microarray profiling: luminal androgen receptor (C1), basal-like with low immune response and high M2-like macrophages (C2), and basal-enriched with high immune response and low M2-like macrophages (C3) [15], with the better outcome being attributed to the C3 cluster. A review of currently developed systems of classification for TNBC is provided by Ahn et al. [16].

These findings all suggest that immunohistochemically defined TNBCs generally display basal-like properties when gene expression is analyzed. In this review, we will focus on discoveries that have been made regarding TNBC, including its basal-like equivalent, as there is an 80% overlap between the two entities [8,9]. However, it is important to mention that these two terms are not synonymous, as they both define molecularly heterogeneous entities.

EPIGENETIC MODIFICATIONS: AN OVERVIEW

Considering the heterogeneity of TNBC, current research is focused on finding new approaches to target this neoplasia; one such strategy employs the use of epigenetics. Being increasingly recognized in all types of cancer, epigenetics plays a major role in tumorigenesis. This field is defined as the study of heritable changes in gene expression without alteration in DNA sequences [17]. Multiple epigenetic modifications with diagnostic, prognostic, or therapeutic significance have already been reported in a number of malignancies, including breast cancer [18].

The first and main epigenetic modifications described and accepted by a large number of authors are DNA methylation and posttranscriptional modifications of histones [17,19,20]. Other more recently described and accepted modifications are noncoding RNAs (ncRNAs) [17-19], as well as chromatin re-

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modeling [19,20], nucleosome positioning, and chromosomal looping [17]. All these markers are strongly interconnected, and one epigenetic modification can easily induce another, as shown in certain circumstances presented later in the article.

DNA methylation is one of the most well-described epigenetic events. Cytosine methylation in CpG islands is a recognized marker of epigenetic silencing and is performed by DNA methyltransferases (DNMTs), among which DNMT1 is responsible for maintaining methylation patterns following replication, and DNMT3a and DNMT3b initiate *de novo* methylation [17].

Histone modifications are covalent posttranslational alterations to histone proteins that influence the chromatin structure and consequent gene transcription; hence, they are important epigenetic markers. Histone modifications include methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation, and they can either activate or deactivate gene expression. Some specific modifications have been correlated with carcinogenic events. Methylation of histone H3 at lysine residues 9 and 27 (H3K9me3, H3K27me3) by the polycomb repressor complex 2 (PRC2) is a hallmark of silenced chromatin [20]. Specific modifications such as lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine trimethylation (H3K4me3), and arginine dimethylation (H4R3me2) are recognized markers of gene activation; on the other hand, lysine methylation (H3K9me2 or H3K9me3 and H4K20me3) is usually associated with gene silencing [21].

ncRNAs do not encode protein, but rather modulate chromatin regulation and gene expression [17]. They include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), and the recently discovered and largely studied long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) [22]. Both lncRNAs and miRNAs are widely studied and have been strongly linked to a large number of diseases.

For didactic reasons, this article has been further divided according to the most relevant types of epigenetic modifications, namely DNA methylation, noncoding RNAs, and histone modifications, as these are the most well-described and widely studied (Supplementary Table 1, available online). However, all these modifications are strongly interconnected, and the regulation of one gene may be the product of more than one epigenetic modification.

DNA METHYLATION

Several studies have looked into the DNA methylation patterns in various breast cancers, including TNBC and its closely related basal-like counterpart.

DNA methylation in triple-negative breast cancer

One of the most comprehensive analyses of the TNBC methylome stratified patient samples into three methylation clusters based on differentially methylated regions (DMRs) [23]. The hypomethylated profile was associated with better survival within the first 5 years post-diagnosis compared with the more heavily methylated subtypes, while the medium methylated cluster was associated with the worst survival. It also identified 17 individual DMRs capable of stratifying TNBC patients into good and poor prognosis groups. Among the genes included are the *WT1* gene and its antisense counterpart, *WT1-AS*, for which high levels of methylation correlated with elevated levels of expression and poor survival. Hypermethylation of the bi-directional promoter is associated with decreased *WT1* and *WT1-AS* expression and improved survival; however, these findings remain to be verified on a larger cohort [23]. The study also described hypermethylation events to mostly occur in CpG islands in the context of global hypomethylation (Figure 2). The hypermethylated regions correlated strongly with the regions of human mammary epithelial cells marked with H3K27me3, a marker of epigenetic silencing. Specifically, 12 methylated genes were identified as both mutated and downregulated; these included *ROBO3* and *SEMA5A* [23], which are genes involved in axon guidance, a pathway that has been newly implicated in tumor initiation and progression in breast cancer [24]. This pathway, originally described in brain development [25], includes the Slit, Netrin, Eph/ephrin, and Semaphorin proteins, which have recently been found to regulate normal mammary development, as well as breast cancer initiation, progression, and angiogenesis [26]. Promoter hypermethylation was found in seven members of this pathway, which may prove to be promising for fu-

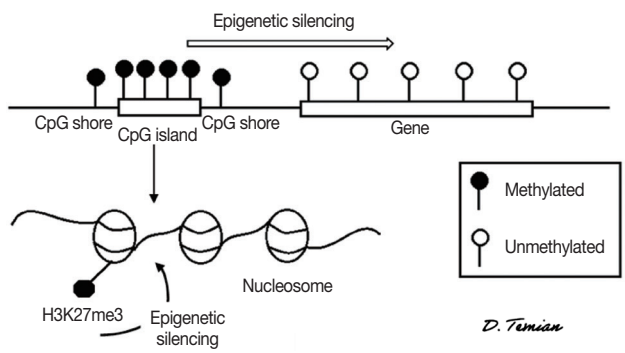

Figure 2. Patterns of methylation described in triple-negative breast cancer. Hypermethylation of CpG islands and shores with hypomethylation of intragenic regions leads to epigenetic silencing. At nucleosomal level the patterns in DNA methylation translate to tri-methylation of lysine 27 on histone H3 (H3K27me3), another marker of epigenetic silencing.

ture investigations in targeted cancer therapy [23].

An earlier study also described a specific methylation pattern for TNBC by analyzing the hypermethylation of 110 CpG islands in 69 cancer-related genes. The TNBC-specific profile was defined by the methylation of five genes (*CD44*, *MGMT*, *CDKN2B*, *RB*, and *p73*) and the non-methylation of 11 genes (*GSTP1*, *PMS2*, *MSH2*, *MLH1*, *MSH3*, *MSH6*, *DLC1*, *CACNA1A*, *CACNA1G*, *TWIST1*, and *ID4*), with *MGMT*, *MMR*, and *ID4* showing the strongest association [27]. Interestingly, there was no significant difference in the methylation of the *BRCA1* and *BRCA2* promoters between triple-negative and non-triple-negative tumors. However, *ID4*, one of the genes in the non-methylated group, is a negative regulator of *BRCA1*; this may imply a new mechanism of *BRCA* silencing that is worth investigating.

DNA methylation and BRCAness in triple-negative breast cancer

Up to 30% of TNBC cases have a *BRCA* mutation [28], and there is a strong association between the two entities, usually leading to a poorer prognosis [29]. However, a large number of tumors share the molecular features of *BRCA*-mutant cancer, a state defined as "BRCAness" [30]. This particular status may be due to the hypermethylation of the promoter region of the *BRCA1* gene [31-33]. There seems to be a mutually exclusive relationship between a *BRCA1* mutation and promoter methylation [31]. Moreover, TNBC with BRCAness may not only benefit from therapy with poly (ADP-ribose) polymerase (PARP) inhibitors and platinum agents [34,35], but also show a survival benefit from anthracycline-based chemotherapy [36].

DNA methylation and triple-negative breast cancer progression

Another whole genome methylation analysis compared the primary tumor to normal adjacent tissues and lymph node metastases, and identified a set of aberrations that may explain the progression of TNBC [37]. Sixteen genes that were identified to be specific to TNBC also had differentially methylated probes, including five genes classified as DMRs—*ANKRD30B*, *COL14A1*, *IGF1*, *IL6ST*, and *MEG3*. An additional set of genes were found to be differentially methylated in the lymph node metastases. Some of these genes correlated with better survival; particularly, the higher methylation of *SPRY2*, *EGR1*, *GREB1*, *ITIH5*, and *LRRC17*, and the low methylation of *AMIGO2*. The same study found that *EGR1* downregulation is inversely correlated with its methylation [37]. Furthermore, a specific gene, *BRMS1*, may epigenetically influence the metastatic potential of TNBC [38]. The expression of *BRMS1* was found to be significantly reduced in TNBC tissue samples and cell lines when compared to normal breast tissue; it was also inversely correlated with lymph node metastasis. DNA methylation-dependent inactivation was proven on breast cancer cell lines (MDA-MB-231, HCC-1937, and MDA-MB-435), a normal breast tissue cell line (MCF-10A), and on primary breast cancer tissues with matched nonmalignant breast tissue [38]. Methylation of this gene significantly correlated with larger size and higher tumor-node-metastasis (TNM) stage of the tumor, suggesting that this gene may function as a tumor suppressor.

DNA methylation and cancer stem cells in triple-negative breast cancer

The role of DNA methylation in TNBC was elucidated by investigating the regulation of breast cancer stem cells (CSC) through promoter methylation [39]. One study found that the promoter regions of *CD44*, *CD133*, and *Musashi-1* (*MSI1*), which are genes associated with stem cell properties [39], were hypomethylated in primary breast cancer samples, and this correlated with a TNBC subtype and a clinically aggressive phenotype.

DNA methylation in basal-like breast cancer

In a study by The Cancer Genome Atlas Network, primary breast cancer tissues were analyzed using multiple platforms, including DNA methylation, exome sequencing, messenger RNA (mRNA) arrays, and miRNA sequencing [40]. There was a high degree of overlap between basal-like and TNBC-defined samples, and the basal-like subtype clustered together most distinctively across all platforms. The study described a hypomethylated phenotype of basal-like tumors, and their findings showed that this subtype correlated with the lowest levels of DNA methylation and up to 80% frequency of *TP53* mutations, as well as being frequently associated with loss of *RB1* and *BRCA*. Interestingly, these findings suggest that basal-like tumors are similar to serous ovarian carcinomas, raising the hypothesis that common therapeutic approaches should be considered [40]. Moreover, the frequency of *BRCA1* and *BRCA2* mutations in these tumors is similar to that of TNBCs, confirming that these subtypes may benefit from PARP inhibitors and platinum compounds.

Specific DNA methylation patterns in basal-like tumors have also been reported in comparison to that in luminal A and B tumors in a study that investigated the methylation profiles of the five intrinsic subtypes of breast cancer [41]. *RASSF1* and *GSTP1*, genes usually associated with the ER+ phenotype [42], were unmethylated in basal-like tumors, in contrast to that of the luminal B phenotype; on the other hand, *ARHGDIB*, *GRB7*, and *SEMA3B* were found to be significantly more methylated in basal-like tumors [41]. Another significant difference observed between *BRCA* mutation carriers is that

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BRCA2 tumors were significantly more methylated than *BRCA1* tumors. Overall, the basal-like phenotypes had lower overall methylation than the other subgroups [41], which supports the findings of the aforementioned studies.

A hypermethylator phenotype has also been described for basal-like breast cancer, namely the CpG island methylator phenotype [23,43]. This does not refer to global hypermethylation but describes concurrent methylation-dependent silencing of a number of genes, including a specific set of genes with predictive power (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *LCN2*, and *SCNN1A*) that are involved in a wide range of malignancies [44]. This specific pattern of methylation may be linked to the overexpression of *DNMT3b*. Moreover, *DNMT3b* seems to be a promising target for TNBC, as shown by the targeted inhibition of *DNMT3b* by RNAi-mediated knockdown in three cell lines (MDA-MB-453, BT549, and Hs578T); all cell lines subsequently showed increased sensitivity to doxorubicin, paclitaxel, and 5-fluorouracil [45].

DNA methylation is one of the most well-studied epigenetic events, and it has also been well-studied in TNBC. However, findings on methylation patterns need to be translated into clinical practice, either by using this data to stratify patients' prognoses and expected outcomes [23,27,37,40] or by further investigating the pathways that have been indicated to show promising results [23,31,40,45].

NONCODING RNAs

Long noncoding RNAs in triple-negative breast cancer

A novel classification scheme for TNBC was established by Liu et al. [46] by integrating the profiles of mRNAs and lncRNAs. Four distinct clusters have been described—IM, LAR, MES, and BLIS—and these partly correlated with the Lehmann subtypes that have been described before [9]; furthermore, the BLIS subtype has been described as the most aggressive phenotype [46].

Further microarray profiling of TNBC has identified a number of lncRNAs with different expression patterns compared to normal tissue [47]. However, the functions, pathway interactions, and importance of these remain to be established. Similarly, another microarray profiling study of lncRNAs in TNBC patient tissue samples found that the dysregulation of the ER in TNBC may be associated with lncRNA *LINC00993* [48]. Recently, another lncRNA, *MALAT1*, was found to play a role in the metastatic potential of TNBC and is reported as a potential prognostic marker for lymph node-negative HER2+ and TNBC [49].

Long noncoding RNAs in basal-like breast cancer

lncRNAs are ncRNAs longer than 200 nucleotides and have various functions in the genome [22]. The expression of lncRNAs in breast cancer has also been investigated using The Cancer Genome Atlas project database [50]. Four clusters were described, with the first being almost entirely populated by the basal-like subtype. *HOTAIRM1* was found to be overexpressed in this cluster; however, its role still remains to be determined [50].

Another promising lncRNA target is *FOXCUT*, which may be a cancer-promoting gene responsible for the aggressive phenotype of basal-like tumors. A study has reported that *FOXCUT* was significantly more highly expressed in basal-like than in non-basal breast cancer subtypes, and that its knockdown inhibited cell migration and proliferation. These data show that *FOXCUT* may be a potential diagnostic and therapeutic marker of basal-like TNBC [51].

A lncRNA linked to aggressive progression in breast cancer through H3K27 methylation [52], *HOTAIR*, has been found to be upregulated in MCF-7-TNR cells, the basal-like derivative of the luminal-like MCF-7 cells. *HOTAIR* and its partner enhancer of zeste homolog 2 (EZH2) form a complex that seems to play a critical role in the maintenance of the basal-like phenotype; when either *HOTAIR* or EZH2 was inhibited, the dysregulated expression of luminal-like and basal-like markers was attenuated and the proliferation of MCF-7-TNR cells was inhibited (Figure 3). *HOTAIR* is also required for the expression of basal-like genes and the proliferation of MDA-MB-157 cells [53]. Furthermore, it was shown that co-target-

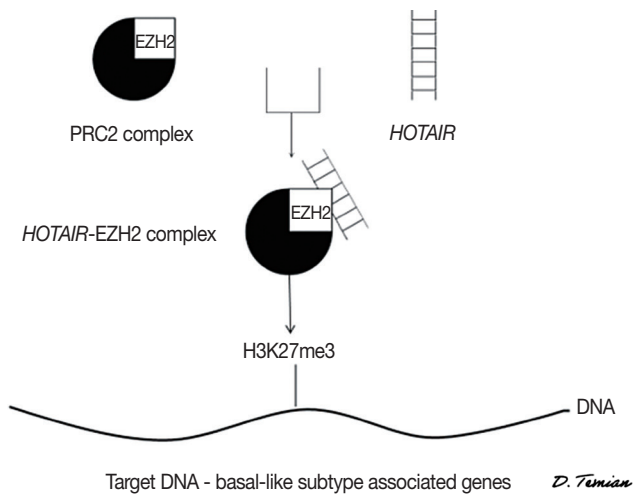

Figure 3. *HOTAIR* in basal-like breast cancer. *HOTAIR* forms a complex with the enhancer of zeste homolog 2 (EZH2) subunit of the polycomb repressor complex 2 (PRC2) complex, which leads to H3K27 trimethylation and maintenance of the basal-like phenotype.
H3K27me3 = tri-methylation of lysine 27 on histone H3.

ing *EGFR* and *c-ABL* in TNBC cell lines through lapatinib and imatinib, respectively, inhibited growth by downregulating *HOTAIR* [54].

MicroRNA in triple-negative breast cancer

miRNAs are small ncRNAs approximately 20 nucleotides long that can posttranscriptionally alter gene expression [22]. Gasparini et al. [55] identified a four-miRNA signature in TNBC that allowed the stratification of patients into high- and low-risk groups. Upregulation of miR-493 and miR-155 correlated with better patient outcome, whereas downregulation of miR-30e and miR-27a correlated with a negative outcome [55].

miRNAs have also been described to be potential TNBC biomarkers. miR-10b, miR-26a, miR-146a, and miR-153 were investigated in breast cancer cell lines and were linked to *BRCA1* expression. In MDA-MB-231 cells, *BRCA1* expression is downregulated by miR-10b and miR-26a. miR-146a is significantly overexpressed in TNBCs without affecting the expression of *BRCA1*, while miR-153 can upregulate *BRCA1* expression in MDA-MB-231 cells [56]. However, Kumaraswamy et al. [57] reported that *BRCA1* expression positively correlates with miR-146a and leads to the downregulation of *EGFR*. Furthermore, Garcia et al. [58] reported that miR-146a and miR-146b-5p downregulate *BRCA1* in TNBC. In a study by Murria et al. [32], miR-590-5p and miR-4417 were found to be hyperexpressed in TNBC. miR-590 can impact ER regulation by interacting with the two mRNA sequences of *ESR1*, while miR-4417 can regulate *BRCA1* mRNA [32].

miRNAs are also drivers of epithelial-to-mesenchymal transition (EMT), an important process in initiating metastasis. An insight into the mechanism that controls their expression in TNBC and correlation to node metastasis was provided by a recent study in which the interaction between two epigenetic mechanisms was shown. miR-200c/miR-141 locus methylation is associated with low miR-200c expression and lymph node invasion in TNBC, favoring metastasis and altering TNBC prognosis [59]. This has also been associated with high levels of ZEB1 transcription factor, which is involved in EMT, suggesting the miR-200c/ZEB1 axis as a possible therapeutic target in metastatic TNBC. Moreover, the mir-200 family of miRNAs has been shown to play important roles in TNBC. Ectopic expression of miR-200b suppressed TNBC migration and metastasis in a mouse mammary xenograft tumor model by inhibiting protein kinase Cα [60]. Another member, miR-200a, has also been shown to modulate TNBC migration by regulating the *EPHA2* oncogene [61], while overexpression of miR-200b-3p and miR-429-5p inhibits the proliferation, migration, and invasion of TNBC cells by inhibiting the LIMK1/CFL1 (LIM domain kinase 1/cofilin 1) pathway [62], thus opening new possibilities for targeted therapies in TNBC.

A comprehensive summary of miRNAs with profiling, functional, prognostic, and therapeutic potential is provided by Mathe et al. [63].

HISTONE MODIFICATIONS

Histone modifications in triple-negative breast cancer

Eight key histone modifications—H3K4me1, H3K4me3, H3K9me3, H3K9ac, H3K27me3, H3K27ac, H3K36me3, and H3K79me2—have been profiled across 13 cell lines, including four TNBCs—MDA-MB-231, MDA-MB-436, MDA-MB-468, and HCC1937 [64]. Subtype-specific histone modification profiles have also been discovered, including distinct H3K36me3 patterns in TNBC cell lines. The androgen receptor (AR) pathway genes were active especially in claudin-low TNBC cell lines, while AR pathway regulators had lower expression levels in basal-like cells lines [64]. Another specific TNBC chromatin state that was identified is the *AFAP1-AS1* marked by the active H3K4me3 and H3K79me2 modifications. The authors reported that this gene has not been linked to TNBC before but is highly expressed and predicts poor prognosis in other cancers, including esophageal adenocarcinoma, pancreatic ductal adenocarcinoma, lung cancer, nasopharyngeal carcinoma, hepatocellular carcinoma, and colorectal cancer; it may also promote tumor invasion by EMT. Small interfering RNA mediated depletion of *AFAP1-AS1* in MDA-MB-231 and HCC1937 cells led to decreased proliferation and colony formation [64].

A transcription factor that has been recently characterized, *BCL11A*, is overexpressed in TNBCs, including basal-like subtypes [65]; it is important for mammary stem and progenitor cells [65] and it promotes tumor formation by interacting with a common subunit (RBBP4/7) of the histone methyltransferase (PRC2) and histone deacetylase (NuRD, SIN3A) complexes [66] to regulate transcription and promote tumorigenesis.

Another family of proteins involved in the epigenetic regulation of gene expression is the bromodomain and extra-terminal (BET) family; they recognize acetylated lysine residues in nucleosomal histones [67,68]. Inhibition of these proteins has been shown to exhibit antitumoral efficacy in solid tumors, including TNBC [68-71]. Many BET inhibitors have shown promising results in preclinical research studies, including synergistic effects with already established therapies [67,70,72,73] and the compound OTX015/MK-8628, is in clinical development for TNBC [67].

Histone modifications seem to play an important role in the

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EMT of TNBC, as reported by a study using the basal-like cell line, MDA-MB-231. The downregulation of histone methyltransferase G9a, histone acetyltransferase *KAT5*, and H3K79 methylator *DOT1L* induce E-cadherin expression and promote an epithelial phenotype with lower migratory and invasive capacity [74]. These findings may prove to be useful insights into using epigenetic targets as means of reducing the risk of metastasis. EMT and mesenchymal state maintenance may also be influenced by a histone 2 variant, macroH2A1. Overexpression of macroH2A1.1 correlated with mesenchymal markers of the claudin-low breast cancer subtype and with poor prognosis in TNBCs [75].

Histone modifications in the basal-like subtype

A histone modification profile specific for breast cancer subtypes was generated from a series of 880 human breast carcinomas [21]. Moderate to low levels of lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine methylation (H3K4me2 and H4K20me3) and arginine methylation (H4R3me2) were reported in carcinomas of poor prognostic subtypes, including basal carcinomas. However, even if the basal-like carcinomas were represented in the low histone modification cluster, the HER2-positive cancers had even lower frequencies of histone modifications [21]. Similar findings were described with regard to a single histone alteration, H3K27me3, that is inversely associated with HER2-positive and basal-like breast cancers [76]. In the latter, H3K27me3 seems to be mediated by a higher expression of *EZH2*, a member of PRC2, leading to histone-mediated silencing of PRC2 target genes [41].

Breast cancer stem cells (BCSC) being important drivers of TNBC aggressiveness is supported by a new study by Li et al. [77], wherein they sorted a population of CD44+/CD24– BCSCs from a culture of MDA-MB-231 cells. Their findings showed that both DNA and histone methylation differed between CSCs and non-CSCs. In particular, H3K4me2 and H3K27me3 were both decreased in CSCs and may have affected both Wnt and GnRH signaling. The sorted CSCs demonstrated greater invasive and tumorigenic capacities both *in vivo* and *in vitro* [77]. However, the exact mechanisms underlying these remain to be elucidated.

Therapeutic potential of histone modifications

Even if histone modification mechanisms in TNBC are still not fully understood, therapies based on these hallmarks already show promising results in preclinical studies. Some of the most widely used epigenetic therapies are based on histone deacetylases (HDACs). These enzymes remove acetyl groups from histones and are thus responsible for regulating gene expression, including tumor suppressors. HDAC inhibitors (HDACi) are currently being investigated in a large number of solid and hematological malignancies, and they have been shown to inhibit tumor growth and induce apoptosis by targeting multiple pathways [78]. A study has shown that HDACi suberoylanilide hydroxamic acid (vorinostat) and sodium butyrate inhibit cell proliferation, induce apoptosis, and downregulate transcription of mutant p53 in TNBC cell lines MDA-MB-231 and BT-549 [79]. Targeting p53 is a strategy that has been successfully investigated in TNBC [80-82]. Similar results have been found for HDACi with panabinostat, which has been shown to induce hyperacetylation of histones H3 and H4, decrease proliferation and survival, and induce apoptosis in TNBC cell lines MDA-MB-157, MDA-MB-231, MDA-MB-468, and BT-549. Panabinostat also decreased tumor size *in vivo* in mice models for the MDA-MB-231 and BT549 lines [83]. Vorinostat enhanced the growth inhibitory ability of PARP inhibitor olaparib in TNBC cells with overexpressed *PTEN*, while *PTEN* knockdown cells were resistant to this combination. The results were confirmed in an *in vivo* MDA-MB-231 mouse model [84].

HDACi may aid in targeting the PD-1/PD-L1 pathway, which regulates T cell function [85]. Several breast cancer cell lines, including TNBC MDA-MB-231, were treated with both class nonspecific (vorinostat and panobinostat) and specific HDACi (valproic acid and entinostat), leading to the upregulation of PD-L1 on tumor cells [86]. A combination of vorinostat and immune checkpoint inhibitors (PD-1 and CTLA-4 blockade) on mice models of TNBC led to decreased tumor growth and prolonged survival. The authors described that vorinostat also promoted Treg downregulation *in vitro* and increased T cells tumor infiltration *in vivo*. This data suggests that HDACi potentiates immune checkpoint inhibitor blockade in TNBC [86].

EMT and metastasis become irreversible when a subpopulation of tumor cells gains the ability to spread from the primary tumor and establish secondary localizations. HDACi entinostat reduces the expression of markers associated with this cell population in TNBC cell lines MDA-MB-231, BT549, and Hs578T; decreases the ability of MDA-MB-231 to form lung metastasis in an *in vivo* mouse model; and reduces tumor formation from patient-derived xenografts [87]. Furthermore, vorinostat also has the ability to prevent brain metastasis of TNBC *in vivo* [88], proving that HDACi should be further investigated for use in the management of metastatic TNBC. Finally, Mekala et al. [89] suggested that HDACi may also aid in re-expression of miRNAs and, by regulating the miR-200 family through HDACi, open a new avenue for research in TNBC.

Other histone-modifying enzymes that can promote aggressiveness of TNBC are histone methyltransferases. A histone methyltransferase, hSETD1A, has been associated with poor outcome and decreased overall survival rates in a retrospective study on 159 TNBC patients [90], indicating that it could be further investigated as a prognostic marker.

OTHER EPIGENETIC CHANGES

These epigenetic changes have an important impact on how genomic DNA is organized, either into tightly packed heterochromatin or as loosely packed euchromatin. DNA methylation, histone modifications, or ncRNAs may recruit protein complexes that indirectly regulate gene expression by how much access to DNA they allow transcription machinery.

Chromatin remodeling refers to the regulation of gene expression by modifying the chromatin architecture to either allow or restrict transcription. This can be done either by post-transcriptional modifications of histones or ncRNAs and their recruitment of the PRC2 complex as previously mentioned, or by ATP-dependent chromatin remodeling protein complexes [19]. SWI/SNF is one of the most well-described complexes with this function [19]. Two ATPases of this complex, *BRG1* and *BRM*, have elevated levels in breast cancer, and their knockdown in a TNBC cell line led to reduced tumor formation *in vivo* and reduced cell proliferation *in vitro* [91,92]. In addition, knockdown of *BRG1* sensitized a TNBC cell line to doxorubicin, 5-fluorouracil, gemcitabine, cisplatin, cyclophosphamide, and paclitaxel [93].

CONCLUSION

TNBC is a heterogeneous oncological entity for which, even if major breakthroughs have been made to describe subtypes with relevance in clinical practice, no specifically designed tool for management exist to date. Epigenetic modifications are currently being intensely studied in all malignant diseases, including breast cancer. TNBC may especially benefit from advances in this domain, considering that no therapeutic targets currently exist for this subtype. We now have at our disposal a multitude of methods to study these epigenetic machinery, and a large amount of data is constantly being generated. Several studies employing epigenetic drugs, namely HDACi, already show promising results. One particular drug, tinostamustine, is a first-in-class alkylating deacetylase inhibitor that combines the DNA-damaging effect of bendamustine with the HDACi vorinostat in a completely new chemical entity [94]. Tinostamustine is currently being tested in a phase I/II clinical trial that also enrolls TNBC patients (NCT03345485). However, the main challenge in translating data into clinical practice still remains. A targeted approached based on identifying which mechanisms drive TNBC in the absence of known receptors and which mechanisms are responsible for its aggressiveness may prove to be a more efficient method for developing new treatments and markers. This malignancy would especially benefit from better classification tools that can identify the patients who can benefit from a certain treatment. The epigenetic field is a very promising area where such answers may be found.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Supplementary Table 1. Current epigenetic modifications in TNBC and basal-like tumors

No.	Findings	Samples	Methods	Ref.
	DNA methylation			
1	- 3 Methylation clusters, with the most hypomethylated associated with better prognosis and the medium methylated with worst prognosis - 17 Potential prognostic regions—lower methylation in low-risk groups - Hypermethylation of genes in axon guidance pathway - Methylation of gene bodies *WT1* and *WT1-AS* vs. promoter methylation	19 Formalin-fixed paraffin-embedded TNBC tissue and 6 matched normal samples	- Whole genome methylation capture sequencing (MBDCap-Seq)	[23]
2	- Methylation of 5 genes (*CDKN2B*, *CD44*, *MGMT*, *RB*, and *p73*) and non-methylation of 11 genes (*GSTP1*, *PMS2*, *MSH2*, *MLH1*, *MSH3*, *MSH6*, *DLC1*, *CACNA1A*, *CACNA1G*, *TWIST1*, and *ID4*) are specific to TNBC	- 61 Breast cancer tissue samples, including 28 TNBC	- Methylation profile of 110 CpGI located within 69 cancer-involved genes (MS-MLPA)	[27]
3	- 27%–37% of TNBC samples show *BRCA1* promoter methylation	377 TNBC samples	- Array Comparative Genomic Hybridisation (aCGH) - *BRCA1* promoter methylation	[31]
4	- *BRCA1* and *ESR1* methylation in TNBC compared to non-TNBC - miR-4417, miR-590-5p higher expression in TNBC	278 Formalin-fixed paraffin-embedded breast cancers containing 79 TNBC	- Promoter methylation (MS-MLPA) of 24 tumor suppressor genes - qRT-PCR for miR expression	[32]
5	- *EGR1* downregulation inversely correlated to methylation - 16 TNBC specific genes show altered DNA methylation, including *IGF1* and *IL6ST* - Higher methylation of *SPRY2*, *EGR1*, *GREB1*, *ITIH5*, *LRRC17* and lower methylation of *AMIGO* are associated with better survival	23 Primary TNBC samples, 12 matched lymph node metastases, 11 matched normal adjacent tissues	- 450 K DNA methylation BeadChip array analysis (Illumina)	[37]
6	- *BRMS1* downregulated by DNA methylation in TNBC cell lines and breast cancer samples - Inverse correlation with lymph node metastasis	- TNBC cell lines MDA-MB-231, HCC-1937, MDA-MB-435 and normal breast tissue MCF-10A - 42 Paired normal and TNBC tissue samples	- RT-PCR - Methylation specific PCR	[38]
7	- Cancer stem cells are regulated by hypomethylation of specific CpG sites of genes associated with stem cell properties *CD44*, *CD133*, and *Musashi-1* (*MSI*), promoter methylation being lower in TNBC	- 4 TNBC cell lines (MDA-MB-231, BT-549, BT-20, and HCC1937) and 5 non-TNBC cell lines (MCF-7, T47D, ZR-75-1, ZR-75-30, and SK-BR-3) - 91 Invasive ductal carcinomas, including 32 TNBC	- Methylation analysis (MassARRAY EpiTYPER sequencing)	[39]
8	- 5 Distinct DNA methylation groups - Group 5–most hypomethylated–associated with basal-like tumors - 80% TP53 mutations in basal-like tumors - Loss of *RB1*, *BRCA1* in basal-like tumors	- Primary breast tumor samples and germline DNA from 825 patients (802 samples for DNA methylation)	- DNA methylation - Exome sequencing - mRNA arrays - miRNA sequencing - Reverse-phase protein arrays	[40]
9	-Specific methylation patterns corresponding to luminal A, B and basal-like subtypes, the most hypomethylated being basal-like and most hypermethylated luminal B - *BRCA2* carriers tumors more methylated than *BRCA1* - *RASSF1*, *GSTP1* unmethylated in basal-like tumors - *ARHGDIB*, *GRB7*, *SEMA3B* methylated in basal-like tumors	- 189 Fresh frozen primary breast tumors and 4 normal breast tissue samples	Array based methylation assay for 1505 CpG loci corresponding to 807 cancer related genes	[41]

(*Continued to the next page*)

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Supplementary Table 1. Continued

No.	Findings	Samples	Methods	Ref.
10	- Methylation of 6 genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *LCN2*, and *SCNN1A*) in basal-like cell lines - Aberrant DNMT3b expression - Elevated total DNA methyltransferase activity	12 Breast cancer cell lines (BT20, BT549, Hs578T, MCF7, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, and ZR-75-1) and normal breast epithelial cell line MCF12A	- Gene expression (RT-PCR), promoter methylation of 64 genes - DNA methyltransferase machinery assessment (total DNMT activity and expression of DNMT1, DNMT3a, and DNMT3b proteins)	[44]
11	- Enhanced effect of doxorubicin, paclitaxel and 5-fluorouracil after DNMT3b inhibition - Re-expression of methylated genes, including *ESR1*	MDA-MB-453, BT549, Hs578T cell lines	- Treatment with 5-aza - RNAi-mediated DNMT3b mediated knockdown and treatment with doxorubicin, paclitaxel and 5-fluorouracil	[45]
	Noncoding RNAs			
12	- TNBC classification by mRNA and lncRNA profiling - 4 clusters: immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal-like (MES) and basal-like and immune suppressed (BLIS)	165 TNBC samples	- Transcriptome profiling (human transcriptome microarrays)	[46]
13	- lncRNAs with differential expression were found in TNBC, with no functional correlations so far	3 Pairs of TNBC and adjacent non-tumor tissues plus 12 paired samples for validation	- lncRNA expression microarray - qRT-PCR validation	[47]
14	- lncRNAs with differential expression were found in TNBC - Possible association between ER, *ANKRD30A* and lnc RNA *LINC0099*	3 Pairs of TNBC and adjacent non-tumor tissues plus 48 paired samples for validation	- lncRNA expression microarray - qRT-PCR validation - Bioinformatics analysis for lncRNA functions (gene ontology)	[48]
15	- lncRNA *MALAT1* promotes metastasis of TNBC and may be useful as a prognostic marker in lymph-node negative patients	- TCGA microarray data set (493 breast cancer samples) - Normal breast cell line MCF10A and breast cancer cell lines -TNBC subtype: MDA-MB-231, Hs578T, HCC1806; HER2+ subtype: SKBR3; luminal subtype: MCF7, T-47D for interrogating functional roles	- MALAT1 expression (qRT-PCR)	[49]
16	- *HOTAIRM1* is upregulated in basal-like tumors	658 Infiltrating breast ductal carcinomas, including 126 basal-like samples (from the TCGA breast cancer RNA-Seq data)	- Bioinformatic analysis	[50]
17	- lncRNA *FOXCUT* is overexpressed in basal-like tumors	- 55 Primary breast cancer samples, including 25 basal-like - MDA-MB-231 and MDA-MB-468 cell lines	- Expression profile (RT-qPCR and siRNA transfection)	[51]
18	- lncRNA *HOTAIR* is up-regulated in MCF-7-TNR cells (basal-like derivative of the luminal-like MCF-7), BT-549 and MDA-MB-157 and plays a role in maintaining the basal-like phenotype	MCF-7-7TNR, MC-7, BT-549 and MDA-MB-157 cell lines	- *HOTAIR* expression and siRNA inhibition	[53]
19	- lncRNA *HOTAIR* expression is repressed by combined treatment of lapatinib plus imatinib through β-catenin downregulation	- MCF-7, T47D, BT474, MDA-MB-468, MDA-MB-231, ZR-75–1, SK-BR3, SUM159 and HCC1806 cell lines - 21 Formalin-fixed paraffin-embedded primary breast tumor tissue, including 11 TNBC	- Lapatinib+imatinib treatment of TNBC cell lines (MDA-MB-231, MDA-MB-468, HCC1806, and SUM159) - *HOTAIR* expression	[54]

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Supplementary Table 1. Continued

No.	Findings	Samples	Methods	Ref.
20	- Upregulation of miR-493 and miR-155 correlate with better outcome - Downregulation of miR-30e and miR27a correlate with poor outcome	173 Paraffin-embedded TNBC samples	- miRNA expression profiling	[55]
21	- miR-10b and miR-26a can downregulate *BRCA1* expression in MDA-MB-231 (TN) and MCF7 (luminal) cell lines	9 Sporadic human breast cancer cell lines of which 7 TNBC and 1 normal breast tissue sample	- miRNA expression profiling	[56]
22	- BRCA1 expression positively correlates with miR-146a and leads to downregulation of *EGFR*	Breast cancer cell lines including 3 TNBC, SKOV3 ovarian cancer cell line and HMLE, MCF10A mammary epithelial cell lines	- miRNA profiling - miR knockdown - Protein expression studies - Mammosphere formation assay	[57]
23	- miR-146a and miR-146b-5p downregulate *BRCA1* in TNBC	- 3 Normal mammary cell lines and 15 breast cancer cell lines, including 3 TNBC - 76 Primary breast tumor tissues - 167 Breast tumor tissues	- miRNA target prediction algorithms - miR-146a/b-5p expression and inhibition studies	[58]
24	- miR-4417, miR-590-5p higher expression in TNBC	278 Formalin-fixed paraffin-embedded breast cancers containing 79 TNBC	- qRT-PCR for miR expression	[32]
25	- miR-200c downregulation correlates with locus methylation and is associated with lymph node metastasis in TNBC - Low levels of miR-200c are associated with high levels of ZEB1 transcription factor which promotes EMT - miR-200c/ZEB1 axis as target for metastatic TNBC	- 51 TNBC samples - TCGA data set - MDA-MB-231 and MDA-MB-157 cell lines for functional analysis	- qRT-PCR and methylation analysis	[59]
26	- miR-200b suppresses TNBC migration and metastasis by inhibiting protein kinase Cα	- MCF-7, T-47D, BT-474, MDA-MB-453, SKBR-3, MDA-MB-468, BT-20, Hs578T and BT-549 cell lines - Mouse mammary xenograft tumor model	- miR-200b expression and knockdown studies - *In vitro* and *in vivo* migration and metastasis studies respectively	[60]
27	- miR-200a modulates TNBC migration through regulating the EPHA2 oncogene	- Breast cancer dataset for mRNA levels of EPHA2 and corresponding patient survival - HC11, MDA-MB-231, SUM159 cell lines	- miR200a transfection study - miRNA expression analysis - Proliferation and migration assays	[61]
28	- Overexpression of miR-200b-3p and miR-429-5p inhibits the proliferation, migration, and invasion of TNBC cell lines	MDA-MB-231 and HCC1937 TNBC cells	- miR transfection - Proliferation, migration and invasion assays	[62]
	Histone modifications			
29	- Distinct H3K36me3 patterns in the TNBC cell lines - AR pathway genes active especially in claudin-low TNBC cell lines, while AR pathway regulators had lower expression levels in basal-like - AFAP1-AS1 found as TNBC specific gene marked by the active H3K4me3 and H3K79me2 modifications	- 2 Normal immortalized cell lines, 76NF2V and MCF10A - 2 Luminal A lines, MCF7, ZR751 - 2 Luminal B lines, MB361, UACC812 - 2 HER2 lines SKBR3, AU565, HCC1954 - 2 Basal TNBC cell lines, MB468 and HCC1937 - 2 Claudin low TNBC cell lines, MB231 and MB436	- ChIP-Seq, GRO-Seq and RNA-Seq analysis - siRNA mediated depletion of AFAP1-AS1 in MDA-MB-231 and HCC1937	[64]

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Supplementary Table 1. Continued

No.	Findings	Samples	Methods	Ref.
30	- BCL11A interacts with histone methyltransferase (PRC2) and histone deacetylase (NuRD and SIN3A) complexes and contributes to maintenance of a chemoresistant breast cancer stem cell population in TNBC including basal-like	- Microarray data sets - Immortalized non-tumourigenic mouse EpH4 and human HMLE cell lines - TNBC cell lines 4T1 (mouse), MDA231, SUM159 and HMLER (human) - Immune compromised mice - BCL11A conditional knockout and knock-in mice	- BCL11A overexpression studies and mammosphere assay - shRNA knockdown of BCL11A - Modified cells' injection to assess tumor formation - TNBC-like tumor promotion by DMBA staining	[65]
31	- *KAT5* (histone acetylase inhibitor), *DOT1L* (H3K79 methylator) and *G9a* (histone methyltransferase) downregulation induce E-CAD expression to promote an epithelial phenotype	- MDA-MB-231 cell line	- siRNA library screening for EMT regulators (729 chromatin modifying targets)	[74]
32	- Overexpression of macroH2A1.1 correlates with claudin-low subtype and TNBC poor outcome	- GEO, EMBL-EBI and publisher databases - MCF-7, MDA-MB231, ZR-75, MDA-MB436 and Hs578T cell lines	- Biostatistical correlation studies on intrinsic molecular subclasses of breast cancer and molecular characteristics of EMT - Protein quantification, qRT-PCR	[75]
33	- 3 Groups of histone modification patterns - Hypomodified cluster, characterized by moderate to low levels of lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine (H3K4me2 and H4K20me3), and arginine methylation (H4R3me2) associated with basal-like and HER2+ subtypes	- 880 Invasive breast carcinomas	- Tissue microarray, immunohistochemistry - Immunofluorescence and western blotting for validation	[21]
34	- Differential H3K4me2 & H3K27me3 methylation between CSC and non-CSC suggest Wnt & GnRH signaling pathways are responsible for aggressiveness in TNBC	- MDA-MB-231 cell line - BALB/c nude+ mice	- Invasion and xenotransplantation assays - RN-seq, WGBS and CHIP-seq analysis	[77]
35	- HDACi suberoylanilide hydroxamic acid (vorinostat) and sodium butyrate inhibit cell proliferation, induce apoptosis and downregulate transcription of mutant p53 in TNBC cell lines	- TNBC cell lines MDA-MB-231 and BT-549	- Transfection studies - Cell cycle and apoptosis assays	[79]
36	- HDACi panabinostat induces hyperacetylation of histones H3 and H4, decreases proliferation and survival, and induces apoptosis in TNBC cell lines and decreases tumor size *in vivo*	- TNBC cell lines MDA-MB-157, MDA-MB-231, MDA-MB-468, BT-549 - Orthotopic MDA-MB-231 and BT-549 mouse xenograft models	- Histone acetylation assays - Proliferation assay and cell cycle analysis - Protein expression studies	[83]
37	- HDACi vorinostat enhances the growth inhibitory ability of PARP inhibitor olaparib in TNBC cells with overexpression of PTEN and *in vivo* in an MDA-MB-231 mouse model	- Human breast cancer cells (MDA-MB-157, -231, -453, -468, BT-549, MCF7, T47D, SK-BR-3, HCC70, HCC1143, and Hs578T) - Breast cancer xenograft mouse model	- Cytotoxic assay and cell cycle analysis - *PTEN* transfection - Proliferation, apoptosis and autophagy analysis	[84]
38	- Combination of vorinostat and immune checkpoint inhibitors (PD-1 and CTLA-4 blockade) on mice models of TNBC lead to decreased tumor growth and prolonged survival	- Human breast cancer cell lines, including 1 TNBC - Mouse breast cancer cells - *In vivo* model of mouse breast cancer cell line similar to TNBC	- PD-L1 expression analysis - Co-culture with peripheral blood mononuclear cells - *In vivo* therapy of mouse model with vorinostat, anti-PD-1 blockade or both drugs	[86]
39	- HDACi entinostat decreases the ability of TNBC to form lung metastasis in an *in vivo* mouse model and reduces tumor formation from patient derived xenografts	- Breast cancer cell lines including 1 TNBC - MDA-MB-231 mouse xenograft - Patient derived xenograft	- Protein and miRNA expression analysis - Mammosphere formation assay - Tumor formation and metastasis development *in vivo*	[87]

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Supplementary Table 1. Continued

No.	Findings	Samples	Methods	Ref.
40	- Vorinostat has the ability to prevent brain metastasis of TNBC *in vivo*	- Mouse model of 231-BR brain trophic subline of the MDA-MB-231 TNBC cell line	- Pharmacokinetic and pharmacodynamic studies of vorinostat uptake in the brain - Histone acetylation, cell cycle and apoptosis analysis *in vitro* and *in vivo*	[88]
41	- Histone methyltransferase hSETD1A positivity correlated with worse outcome	- 159 TNBC samples	- Protein expression studies—immunohistochemistry, qRT-PCR	[90]

TNBC=triple-negative breast cancer; RT-PCR=reverse transcription polymerase chain reaction; qRT-PCR=quantitative RT-PCR; mRNA=messenger RNA; miRNA=microRNA; RNAi=RNA interference; lncRNA=long noncoding RNA; ER=estrogen receptor; TCGA=The Cancer Genome Atlas; siRNA=small interfering RNA; EMT=epithelial-to-mesenchymal transition; AR=androgen receptor; HER2=human epidermal growth factor receptor 2; shRNA=small hairpin RNA; DMBA=7,12-dimethylbenz(a)anthracene – a potent carcinogen; CSC=cancer stem cells; HDACi=histone deacetylase inhibitors; PARP=poly (ADP-ribose) polymerase.

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