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Integrative Molecular Analysis of DNA Methylation Dynamics Unveils Molecules with Prognostic Potential in Breast Cancer

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Abstract: DNA methylation acts as a major epigenetic modification in mammals, characterized by the transfer of a methyl group to a cytosine. DNA methylation plays a pivotal role in regulating normal development, and misregulation in cells leads to an abnormal phenotype as is seen in several cancers. Any mutations or expression anomalies of genes encoding regulators of DNA methylation may lead to abnormal expression of critical molecules. A comprehensive genomic study encompassing all the genes related to DNA methylation regulation in relation to breast cancer is lacking. We used genomic and transcriptomic datasets from the Cancer Genome Atlas (TGCA) Pan-Cancer Atlas, Genotype-Tissue Expression (GTEx) and microarray platforms and conducted in silico analysis of all the genes related to DNA methylation with respect to writing, reading and erasing this epigenetic mark. Analysis of mutations was conducted using cBioportal, while Xena and KMPlot were utilized for expression changes and patient survival, respectively. Our study identified multiple mutations in the genes encoding regulators of DNA methylation. The expression profiling of these showed significant differences between normal and disease tissues. Moreover, deregulated expression of some of the genes, namely *DNMT3B*, *MBD1*, *MBD6*, *BAZ2B*, *ZBTB38*, *KLF4*, *TET2* and *TDG*, was correlated with patient prognosis. The current study, to our best knowledge, is the first to provide a comprehensive molecular and genetic profile of DNA methylation machinery genes in breast cancer and identifies DNA methylation machinery as an important determinant of the disease progression. The findings of this study will advance our understanding of the etiology of the disease and may serve to identify alternative targets for novel therapeutic strategies in cancer.

Keywords: DNA methylation; epigenetic modification; breast cancer; genomics; in silico analysis

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1. Introduction

Among the various types of epigenetic modifications of the genome, DNA methylation is the first and highly characterized one that involves addition of a methyl group to the fifth carbon of the cytosine residue at CpG dinucleotides [1–3]. DNA methylation is carried out by the action of a group of enzymes called DNA methyltransferases (DNMTs), which are also termed as writers of this modification and are conserved in various species including bacteria, plants and mammals [4]. DNA methylation has a critical role in regulating the gene

expression in various physiological processes; for example, in X-chromosome inactivation, genomic imprinting, repetitive elements repression and silencing of potentially active transposable elements [5,6]. Various techniques have been developed for detection of DNA methylation, including sodium bisulfite conversion, methylation-sensitive restriction enzymes and methylated DNA immunoprecipitation. The choice of DNA methylation detection technique depends on several factors such as the scale of analysis (gene-specific vs. genome-wide), the resolution required and the availability of resources [7]. While methylation at the CpG sites is the most frequent one, recent studies in the brain and embryonic stem cells have identified methylation beyond the CpG context [8,9]. Dynamic regulation of genome methylation is a general attribute of early mammalian development that is characterized by highly regulated methylation and demethylation of lineage specific genes [10,11]. The dynamics of DNA methylation during early development necessitates the high expression of DNA methyltransferases such as DNMT3A and DNMT3B, which are de novo methyltransferases, and DNMT1, which is maintenance methyltransferase [12,13].

DNA methylation in gene promoters affects gene expression by regulating the recruitment of methylated DNA binding proteins (MBPs), also called readers of DNA methylation, which influence binding of transcription factor and overall chromatin structure [14]. Methyl CpG binding protein 2 (MeCP2) was the first methylated DNA binding protein that was identified by its ability to bind DNA sequences when they are methylated at CpG [3]. MeCP2 contains a methyl-CpG-binding domain (MBD) that comprises 70–85 amino acids [15] that was used to identify other proteins with methylated DNA ability to bind [16]. To date, a number of methyl-CpG binding proteins have been identified that are classified based on methylated DNA binding domains [17]. After binding to the methylated DNA, MBPs relay the DNA methylation signal into various functional states through interactions with diverse partners that eventually regulate gene expression by specific mechanisms, such as chromatin organization and epigenome maintenance [18].

DNA methylation is considered relatively stable epigenetic modification compared with most histone modifications; however, active loss of DNA methylation was mechanistically described recently. The discovery of ten eleven translocation protein 1 (TET1) and its ability to modify methylcytosine and remove methyl group from DNA provided initial insights regarding active demethylation [19]. TET1 belongs to a family of three enzymes namely TET1, TET2 and TET3, that are involved in the stepwise oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [20,21]. These enzymes are collectively called “erasers” of DNA methylation.

One of the hallmarks of cancer is the disruption of epigenetic profiles including DNA methylation patterns. Abnormal DNA methylation is one of the most critical epigenetic factors directly linked with tumorigenesis, because aberrant methylation can contribute to transcriptional changes of tumor suppressors and oncogenes [22–24]. Disruption in the activities of members of “readers”, “writers” and “erasers” of DNA methylation has been reported [25,26]. DNA methyltransferases coordinate mRNA expression in normal tissue and tumors [27]. Moreover, elevated expressions of DNMTs have been reported for hepatic, prostate and breast cancers [28–31].

Although multiple reports that have focused on the role of one or a few regulators of DNA methylation in cancer progression have significantly enhanced our understanding of the role of this important epigenetic modification in cancer [32–34], to our knowledge a comprehensive study that provides overarching molecular and genetic profile of DNA methylation machinery is lacking. This study provides a genomic and molecular landscape of writers, readers and erasers of DNA methylation in breast cancer. The majority of molecules show significant expression anomalies in breast cancer. Additionally, we identify that deregulated expression of some of the molecules have prognostic potential in breast cancer.

2. Materials and Methods

2.1. Writers, Readers and Erasers of DNA Methylation

The genes coding for writers, readers and erasers of DNA methylation are listed in Table 1. The list was compiled using data from earlier studies [17,35]. All the genes listed in the table were used for mutational analysis, expression changes and patient overall survival.

Table 1. This is a table showing list of genes encoding for writers, readers and erasers used in the study.

Category	Gene Symbol	Protein Function
Writer	DNMT1	Maintenance methylation
	DNMT3A	De novo methylation
	DNMT3B	De novo methylation
	DNMT3L	Assists the de novo methyltransferases
Reader	MeCP2	Methylated DNA binding
	MBD1	Methylated DNA binding
	MBD2	Methylated DNA binding
	MBD3	Methylated DNA binding
	MBD4	Methylated DNA binding
	MBD5	Methylated DNA binding
	MBD6	Methylated DNA binding
	BAZ2A	Methylated DNA binding
	BAZ2B	Methylated DNA binding
	SETDB1	Methylated DNA binding
	SETDB2	Methylated DNA binding
	UHRF1	Methylated DNA binding
	UHRF2	Methylated DNA binding
	ZBTB33	Methylated DNA binding
	ZBTB4	Methylated DNA binding
	ZBTB38	Methylated DNA binding
	ZFP57	Methylated DNA binding
	KLF4	Methylated DNA binding
	EGR1	Methylated DNA binding
	WT1	Methylated DNA binding
	CTCF	Methylated DNA binding
Eraser	TET1	DNA demethylation
	TET2	DNA demethylation
	TET3	DNA demethylation
	TDG	DNA demethylation

2.2. Genetic Alterations

To identify mutations in various genes encoding DNA methylation regulators, we utilized the Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga, accessed on 10 April 2022) data using cBioportal [36,37]. Breast cancer datasets from multiple sources are accessible at cBioportal. We selected the latest "TCGA Breast Invasive Carcinoma (TCGA, Pan-Cancer Atlas)" dataset that consisted of 996 patient samples with copy-number alterations (CNAs) and mutation data. Therefore, following genetic alterations were

identified in these 996 breast cancer patients' datasets: (1) copy-number alterations (CNAs) and (2) mutations.

2.3. Changes in the Transcriptome

We employed Xena to compare transcriptome changes between normal tissue and breast cancer samples [38], which assimilates data from TCGA and GTEX to draw a comparison [39]. The heat map was generated using the gene list in Table 1. After launching Xena, all the genes in the list were incorporated. We selected the first variable phenotype "main category" and "gene expression" as the second genomic variable. All the genes encoding writers, readers and erasers of DNA methylation were incorporated to generate the heatmap. The transcription changes in the individual genes were obtained from the "view chart" tool to generate box plots that compare GTEX and TCGA breast cancer patients' datasets. We used Xena specifically for generating heatmap and box plots because Xena employs the same pipeline for TCGA and GTEx samples to eradicate batch effects [38].

2.4. Survival Analysis

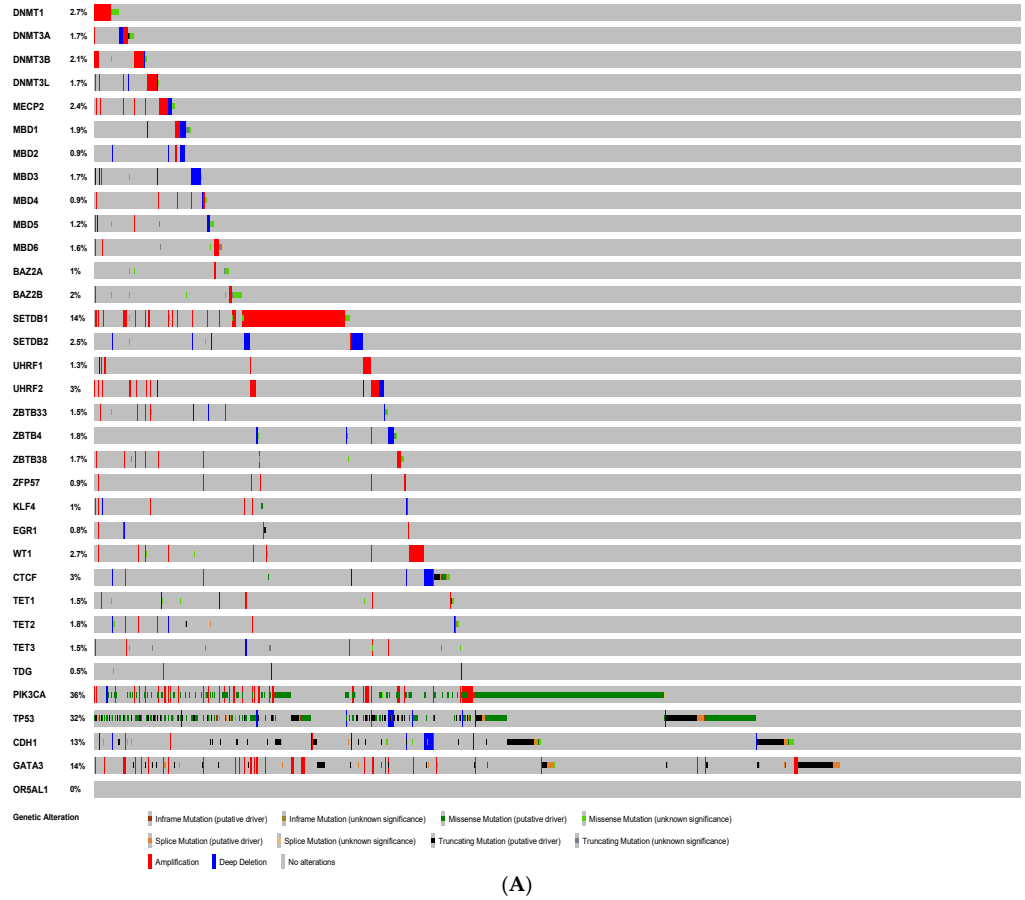
The Kaplan–Meier Plotter [40] was used to generate Kaplan–Meier plots utilizing multiple microarray expression datasets from 7462 breast cancer patients. For overall survival (OS), the analysis was run on 2879 breast cancer patients that were split by median. For the best probe selection, "Jetset" was selected, and patient survival was analyzed for the indicated time spans [40].

3. Results

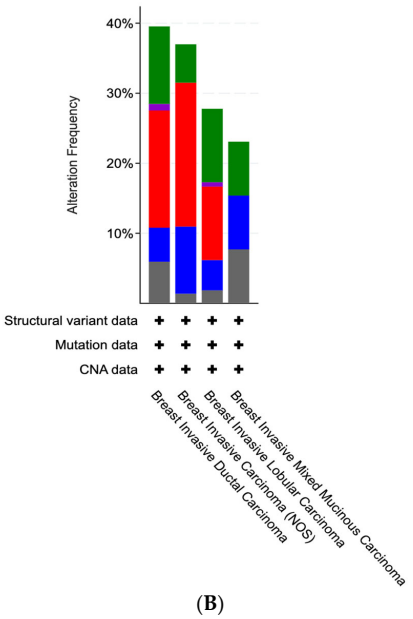
3.1. Mutational Analysis of DNA Methylation Regulators in Breast Cancer

In our previous study on the genomic analysis of TCGA datasets, we reported that several genes encoding molecules related to nucleocytoplasmic shuttling were mutated [41]. In the current study, using cBioportal and selecting breast invasive carcinoma (TCGA, Firehose Legacy) datasets, we carried out mutational analysis of the genes encoding proteins corresponding to writers, readers and erasers of the DNA methylation. The characterization of TCGA datasets using cBioportal led to the identification of various mutations in breast cancer patients. The frequency genetic alteration for various genes differed markedly (ranging from 0.6% in *EGR1* to 11% in *SETDB1*) (Figure 1A) amongst various genes under study. The most prevalent genetic alteration was found to be the gene amplification. Additional mutations detected were deep deletions, base substitutions and multiple alterations. Interestingly, alteration frequency in all the genes related to DNA methylation was found to be in 371 (37%) of queried patients, representing significance of DNA methylation dynamics (Figure 1), though with variable rates and mutation types, genetic alterations in these genes were detected in all breast cancer subtypes. We used *PIK3CA, TP53, CDH1* and *GATA3* genes as controls to validate our analysis. Consistent with the literature, higher mutation rates were found in these genes (Figure 1A). One of the olfactory receptor genes, *OR5AL1*, with no known connection with breast cancer, was used as a negative control.

We stratified breast cancer patients into subtypes and analyzed mutation frequency. Alteration frequency was variable depending on subtypes, and amplification was non-existent in breast invasive mixed mucinous carcinoma (Figure 1B). Interestingly, the genetic alterations in DNA methylation machinery were not specific to breast cancer. Querying 10,953 patients/10,967 samples in 32 studies from TCGA Pan-Cancer Atlas Studies identified comparable trends in mutations in the majority of cancers. This reveals that mutations in the genes coding for writers, readers and erasers of DNA methylation span a spectrum of cancers (Figure 2).



(**A**)



(**B**)

Figure 1. Genes encoding writers, readers and erasers of DNA methylation show genetic alterations in breast cancer. (**A**) Frequency of genetic alterations in these genes are in breast cancer patients. Individual lines in the horizontal bars are 996 breast cancer patients. Color schemes in the figure correspond to various genetic alterations including missense mutation, deep deletion, amplification, in frame mutation and truncating mutations. *PIK3CA*, *TP53*, *CDH1* and *GATA3* are used as positive controls, while *OR5AL1* is used as a negative control with 0% mutation rate. (**B**) Subtypes of breast cancer showing various genetic alterations in DNA methylation regulators genes.

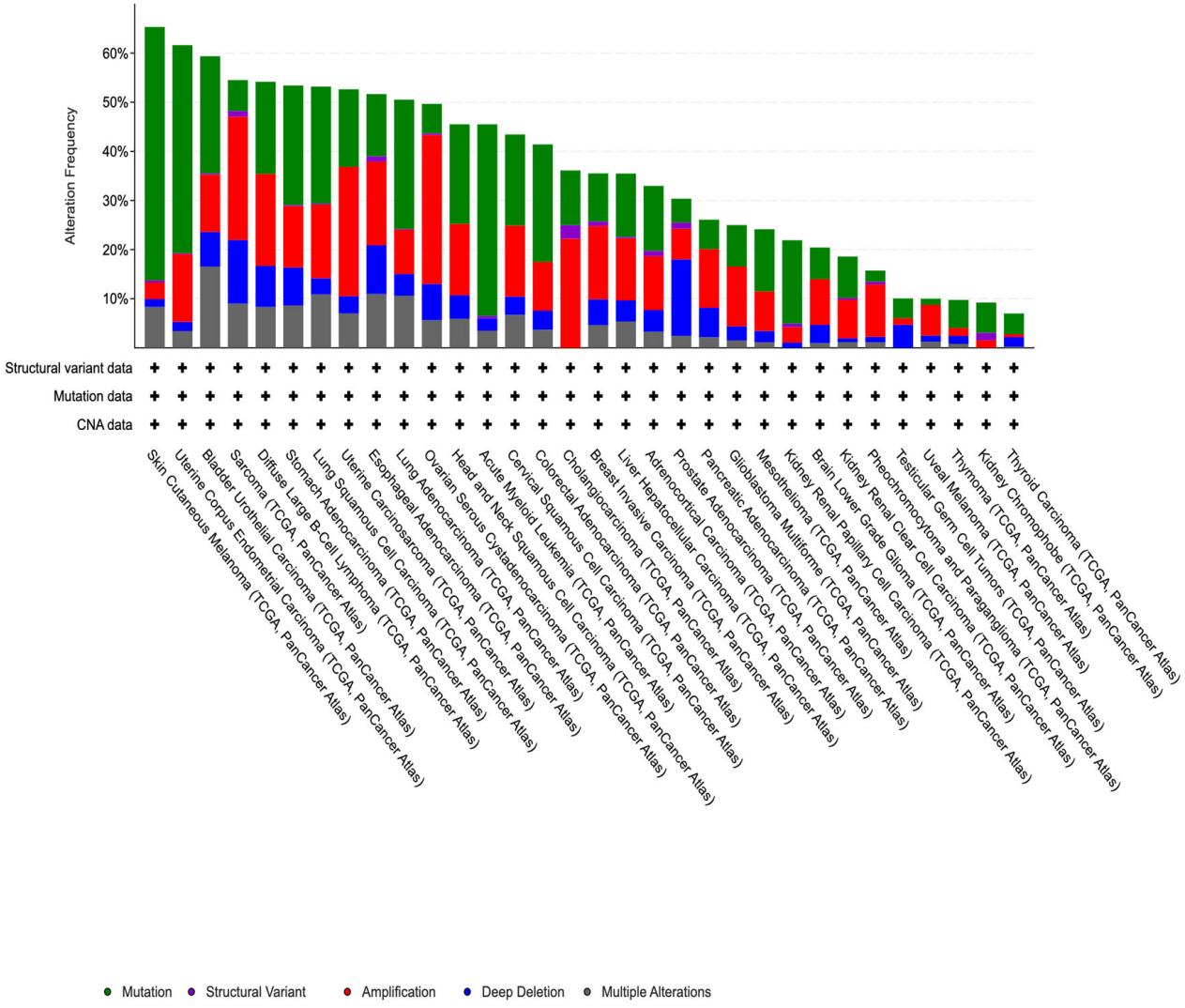


Figure 2. Frequency of genetic alterations in genes encoding DNA methylation regulators in a wide variety of cancers. TCGA Pan-Cancer Atlas datasets employing 10,953 patients/10,967 samples in 32 studies. Genes encoding DNA methylation regulators are mutated in 4213 (38%) of samples.

3.2. Expression Anomalies in Breast Cancer

Recent studies have shown that transcriptomic anomalies underlie tumor development. Aberrant gene expression may potentially serve as a biomarker in breast cancer. Therefore, we analyzed transcripts of all the genes under study in the TCGA and GTEx datasets. As can be seen in Figure 3A, several genes under study showed significant expression deregulation in breast cancer. There has been a mixed trend regarding over- or under-expression of DNA-methylation-related genes in cancer patients compared with normal tissue samples. *MBD1* and *MBD3* showed clear loss of expression, and this trend correlated with high rates of deletions as shown in Figure 1A. Similarly, overexpression of *UHRF1* correlated with genetic amplification. Figure 3B shows expression changes in the individual genes between TCGA and GTEx datasets. Overall, our results show that the majority of genes encoding DNA methylation writers, readers and erasers go through massive expression deregulation in breast cancer patients.

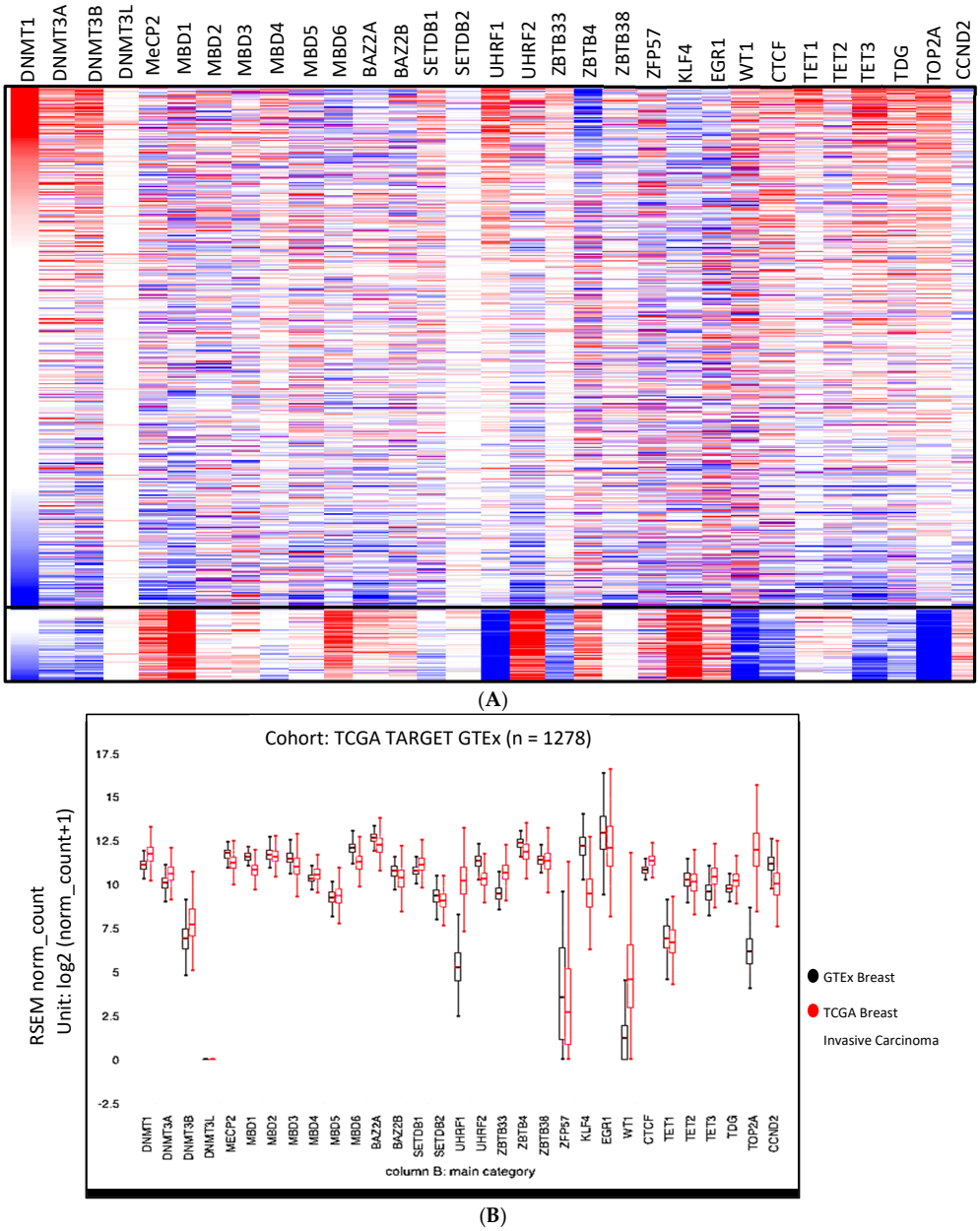


Figure 3. Expression level of genes that encode writers, readers and erasers of DNA methylation. (**A**) Red/blue bars represent over/under-expression of the genes under study. Expression levels are compared between GTEx and TCGA datasets. As control, TOP2A and CCND2 are employed. (**B**) Expression comparison of individual gene transcripts in normal vs. breast cancer patients. Welch's *t*-test was used to calculate the *p*-values for individual genes. DNMT1 $p = 5.781 \times 10^{-74}$ (t = −22.11), DNMT3A $p = 1.981 \times 10^{-39}$ (t = −15.12), DNMT3B $p = 1.076 \times 10^{-33}$ (t = −13.78), DNMT3L $p = 0.07770$ (t = −1.770), MECP2 $p = 0.000$ (t = 17.54), MBD1 $p = 0.000$ (t = 34.01), MBD2 $p = 0.00002861$ (t = 4.248), MBD3p = 0.000 (t = 12.02), MBD4 $p = 4.561 \times 10^{-30}$ (t = −12.52), MBD5 $p = 0.1683$ (t = −1.381), MBD6 $p = 0.000$ (t = 23.56), BAZ2A $p = 0.000$ (t = 13.08), BAZ2B $p = 0.000$ (t = 10.07), SETDB1 $p = 1.064 \times 10^{-31}$ (t = −12.98), SETDB2 $p = 2.190 \times 10^{-8}$ (t = 5.781), UHRF1 $p = 6.499 \times 10^{-120}$ (t = −47.29), UHRF2 $p = 0.000$ (t = 29.62), ZBTB33 $p = 4.126 \times 10^{-94}$ (t = −30.02), ZBTB4 $p = 0.000$ (t = 17.21), ZBTB38 $p = 0.005314$ (t = 2.800), ZFP57 $p = 0.05441$ (t = 1.933), KLF4 $p = 0.000$ (t = 37.18), EGR1 $p = 7.124 \times 10^{-11}$ (t = 6.803), WT1 $p = 1.442 \times 10^{-106}$ (t = −28.61), CTCF $p = 4.217 \times 10^{-65}$ (t = −21.70), TET1 $p = 0.01331$ (t = 2.491), TET2 $p = 0.06953$ (t = 1.821), TET3 $p = 5.981 \times 10^{-48}$ (t = −17.60), TDG $p = 5.148 \times 10^{-49}$ (t = −17.12), TOP2A $p = 6.588 \times 10^{-156}$ (t = −58.62), CCND2 $p = 0.000$ (t = 20.99).

3.3. DNA Methylation and Patient Overall Survival

After establishing that genetic alterations and expression changes have significantly high prevalence in cancer patients, we next determined whether these changes in gene expression have prognostic potential. The Kaplan–Meier (KM) Plotter [40] has integrated microarray datasets that can be used to calculate overall survival (OS) of breast cancer patients according to expression levels of DNA methylated related genes. The current analysis was run on 2879 breast cancer patients. We tested all the genes and found that overexpression of *MBD1, MBD6, BAZ2B, ZBTZ3B, KLF4* and *TET2* correlated with better overall survival, while *DNMT3B* and *TDG* overexpression were correlated with poor patient overall survival (Figure 4). *TOP2A* was used as a control in our analysis, and, consistent with its published role as a marker for poor patient survival, the results were replicated in our analysis. The other genes under study were not significantly correlated with patient survival (Supplementary Figure S1). Collectively, our analysis identified several molecules regulating DNA methylation dynamics that might have potential for prognostication in breast cancer.

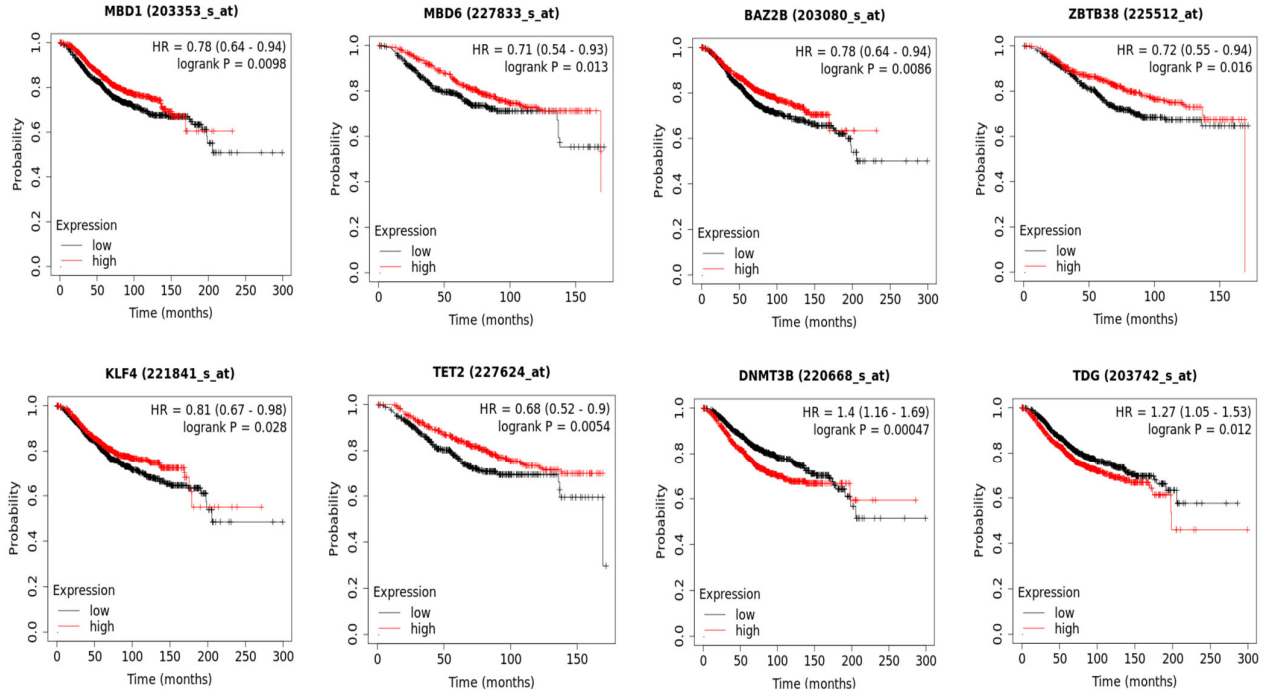


Figure 4. Expression of genes encoding DNA methylation machinery correlate with breast cancer patients' overall survival. Kaplan–Meier plots indicate patient overall survival. The *X* axis denotes patient overall survival percentage, while the *Y* axis represents months after the diagnosis. Black color represents the low expression group, while the high expression group is indicated by the red color. The patients were split by median. The log-rank test is used to determine *p*-values.

4. Discussion

In the current study, a comprehensive genomic and molecular landscape of genes encoding writers, readers and erasers of DNA methylation in breast cancer is provided. Our data show that these genes are not only genetically altered but show expression anomalies in breast cancer patients. Almost all the genes in the above categories are genetically altered to varying degrees. Mutations and expression deregulations in these genes may have a variety of effects on various signaling pathways. As myriads of genes are regulated by DNA methylation, alterations in DNA methylation regulators have far-reaching consequences.

The role of DNA methylation in cancer became apparent when the role of DNA methylation in the inactivation of tumor suppressor genes was revealed [42]. While earlier studies mainly focused on the genetic aspects of cancer, the epigenetic regulation in cancer

became pivotal with our understanding of DNA methylation in cancer development [43] and its connection with gene regulation quantitatively. DNMT1, DNMT3A and DNMT3B are the major DNA methyltransferases, but, surprisingly, the mutation rates in these genes remained low (Figure 1). However, consistent with other studies, we found major expression elevations in all these methyltransferases that may contribute to significant alterations in DNA methylation [44–47].

In addition to changes in DNA methylation, equally important is the mechanisms that relay the DNA methylation information. The methylation marks are read and interpreted by the action of "reader" proteins that can recognize and bind to the methylated DNA regions. Out of all the readers under study, *SETDB1* showed highest levels of genetic alterations with 11% of patients having gene amplifications. However, other readers had massive expression deregulations, mostly showing loss of expression, despite having relatively low representation in genetic alterations. Diversity in proteins that interpret methylation signals indicates the variation of the downstream signaling, e.g., MBD1 directs histone methylation to sites of DNA methylation, MeCP2 is involved in heterochromatin clustering, MBD2-3 dictate transcriptional silencing through NuRD/Mi-2 complexes, MBD4 maintains correct DNA methylation by suppressing CpG mutability, SETDB1 is involved in heterochromatin formation and BAZ2A is a component of the nucleolar remodeling complex NoRC [18]. Additional functions of MBPs have also been identified that are independent of methylated DNA binding. ChIP-seq studies for both MBD3 and MBD2 have shown that both proteins bind at transcriptionally active and unmethylated promoters [48,49].

The erasers of DNA methylation also showed dramatic expression differences. Consistent with earlier studies, *TET1* and *TET2* were modestly downregulated [50,51]. However, *TET3* and *TDG* showed massive upregulation in the TCGA data. Differential regulation of *TET* and *TDG* also had opposing outcomes. While *TET2* expression was correlated with better overall survival, *TDG* overexpression correlated with poor patient survival. *TET2* has been reported to act as an anti-tumor factor, and its decreased expression has been associated with solid malignant tumors, such as colorectal, gastric, ovarian, prostate and breast cancers [52–56]. TET2 regulates the PD-L1 gene in breast cancer cells without altering the DNA methylation and hydroxymethylation (hmC) level at the promoter region pointing to functions that are independent of DNA demethylation [56]. *TDG*, on the other hand, has contradictory roles. Based on its ability to potentiate p53 signaling [57] and positively regulate p15 (ink4b) expression [58], it was suggested to act as a tumor suppressor. However, it is overexpressed in human colorectal cancer (CRC) patients, and knocking down *TDG* in several CRC cell lines inhibited cell growth [59]. Moreover, *TDG* knockdown suppressed tumor formation of melanoma cell lines in xenograft models, indicating that *TDG* activity is important for tumorigenesis [60]. Our data evince its potential oncogenic function in breast cancer.

DNMT3B was the only "writer" of DNA methylation that was not only overexpressed but also had poor prognostication. This is consistent with earlier studies wherein DNMT3b was proposed to be a prognostic biomarker for breast cancer [33]. Interestingly, the majority of the readers of DNA methylation showed better overall survival that included *MBD1, MBD6, BAZ2B, ZBTB38* and *KLF4.* While the functions of many DNA methylation readers are now clear, more research is needed as some of these proteins are still less explored [17]. Future work on these candidates will unravel their anti-tumorigenic functions and underlying mechanisms. Molecular studies using cellular and xenograft models are required to understand the functions of these molecules.

5. Conclusions

In conclusion, the role of DNA methylation regulation in cancer therapeutics has gained attention in recent years after their role became apparent in various tumors. Loss of function studies have further substantiated their potential as therapeutic targets. Currently, FDA-approved DNMTis include 5-aza-2′-deoxycytidine (decitabine) and 5-azacytidine, which are cytidine analogs with a nitrogen atom replacing carbon in the fifth position of the

ring. However, the lack of specificity of these therapeutic agents remains a major challenge. To overcome this challenge, the combination of traditional treatments with epigenetic drugs and the latest genomic tools is necessary to confer target specificity. Additionally, our study has identified several molecules such as *DNMT3B*, *MBD1*, *MBD6*, *BAZ2B*, *ZBTB38*, *KLF4*, *TET2* and *TDG* in the DNA methylation pathway that have potential prognostic roles. This is the first study to unravel a comprehensive molecular and genetic profile of DNA methylation machinery genes in breast cancer and identifies DNA methylation machinery as an important determinant of disease progression. However, future research is needed to validate and target these molecules using cellular and animal models. These efforts will advance our understanding of the underlying mechanisms of DNA methylation in cancer and pave the way for the development of novel targeted therapeutic strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedinformatics3020029/s1, Figure S1.

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