GMR
The Original

EPIGENETIC PROFILING: ANALYSIS OF DNA METHYLATION AND DEMETHYLATION PATTERNS IN TRIPLE-NEGATIVE BREAST CANCER

Dr. Supriya R Aland[1], Dr. Indira A. Hundekari[2*], Dr. P J Yaranal[3]

[1] Tutor, Dept of Biochemistry, S Nijalingappa Medical College, HSK Hospital and Research Centre, Bagalkot, 587102 (Karnataka) IND
[2*] Professor, Dept of Biochemistry, Bijapur Lingayat District Educational (BLDE) Shri B.M. Patil Medical College Hospital and Research Centre, Vijayapura, 586103 (Karnataka), IND
[3] Processor and HOD, Dept of Pathology, S Nijalingappa Medical College, HSK Hospital and Research Centre, Bagalkot, 587102 (Karnataka) IND
*Corresponding Author: Dr. Indira A. Hundekari, indira.hundekari@bldedu.ac.in

ABSTRACT

Introduction: TNBC is a highly advanced, molecular subtype of breast cancer, characterized by lack of gene expression of the estrogen receptor, progesterone receptor and HEGF-2 (human epidermal growth factor receptor-2). Additionally, patients with TNBC, the absence of particular therapeutic targets are associated with unfavorable clinical results, while the high risk of metastasis and recurrence. There is increasing evidence that epigenetic modifications, such as methylation of DNA and demethylation events, are important factors in the spread and development of TNBC, also in metastasis and treatment resistance. While active demethylation of DNA through 5-hydroxymethyl-cytosine (5-hmC) has been connected to chromatin remodeling and gene regulation, DNA methylation through 5-methyl-cytosine (5-mC) is a crucial mechanism for silencing genes. Tumor suppressor genes, oncogenic signaling pathways, and all cellular differentiation pathways have been linked to the abnormal levels of 5-mC and 5-hmC found in TNBC samples.

Method: The research investigated how DNA methylation and demethylation markers in tumor tissue affect the response of TNBC patients to neoadjuvant chemotherapy (NACT) compared to those who did not receive NACT, while exploring the relationship between 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC), revealing new insights into the predictive significance of these epigenetic indicators. The study comprised 40 female patients with TNBC, where 15 underwent neoadjuvant chemotherapy (NACT) and 25 did not. Global DNA methylation and demethylation levels were measured using an ELISA-based technique to investigate the content of 5-mC and 5-hmC levels in DNA extracted from biopsy samples of patients receiving NACT and postoperative tissues from those without NACT.

Results: There was a significant positive correlation found between two global methylation markers ($rs=0.929$, $p < 0.0001$).Favorable association between 5-hmc and 5-mC was observed ($rs= 0.017$, $p < 0.951$ and $rs= 0.069$, $p < 0.743$, respectively). Hence we propose that 5-methylcytosine and 5-hydroxymethylcytosine to be consider as key molecular markers of pathogenesis of TNBC. Moreover, the findings support epigenetic interventions aimed to restore DNA methylation dynamics to a "normal" state.

CATEGORIES: General Surgery, Pathology, Oncology

KEYWORDS: DNA methylation and demethylation, Triple-negative breast cancer, epigenetics, Ten-eleven translocases, DNA Methyl Transferases (DNMTs), methylome profiling

1. INTRODUCTION

1.1 TNBC

Breast cancer is the most prevalent cancer in women worldwide, and a leading cause of mortality in women [1]. The molecular subtype triple-negative breast cancer, which represents about 15-20% of all type of breast cancers, and is an aggressive clinical phenotype [2].TNBC is characterized by the lack of expression of human epidermal growth factor receptor-2 (HER2), progesterone receptor (PR), and estrogen receptor (ER) [3].This molecular signature makes it possible to use some of the targeted endocrine treatments and HER2 targeting therapies, and offers few options. TNBC is known to have high tumor size, genomic instability, distant metastasis and tumor recurrence. Patients with larger tumors, a higher grade and worse prognosis compared to other type of breast cancer [4]. Additionally, the therapeutic management of TNBC is further complicated by its biological heterogeneity, and new molecular mechanisms contributing to the progression of the disease need to be identified [5].Epigenetic changes have been highlighted in recent studies as important in the pathogenesis of TNBC. In contrast to mutations, epigenetic changes may be reversible, which opens the opportunity to the discovery of biomarkers and therapeutic intervention [1].

1.2 Epigenetic Regulation in Cancer

Epigenetic is the study of changes in gene expression that occur without altering the DNA sequence [6]. Components of epigenetic mechanisms are— DNA methylation, chromatin remodeling, histone modifications and non-coding RNA chromatin regulation [7]. These processes act together to control the activity of transcription and cell identity. One example of an epigenetic alteration that has been extensively studied is DNA methylation. The methylation occurs at the 5th carbon of cytosine residues that are the part of CpG dinucleotide, creating 5-methylcytosine [8]. Gene silencing, genomic stability, X-chromosome inactivation and DNA methylation is linked to the repression of repetitive sequences [9].In normal tissues, methylation of DNA is well regulated. However, in most cases there exists a general methylation defect, hypomethylation

of the whole genome and hypermethylation of tumor suppressor gene promoter regions. These alterations are all associated with oncogene activation. Chromosomal instability and malignant transformation [10].

1.3 Biological Significance of 5-Methylcytosine

In mammals, epigenetic alteration most commonly associated with transcriptional regulation is cytosine methylation [8]. DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) have the ability to methylate cytosine using S-adenosylmethionine. The methylation pattern is maintained by DNMT1 during DNA replication, and the establishment of the methylation pattern involves DNMT3A and DNMT3B during development and differentiation. DNA methyltransferases are overexpressed in a number of malignancies such as liver cancer, leukemia and breast cancer [11]. In TNBC, abnormal 5-mC deposition has been linked to the repression of numerous significant tumor suppressor genes which regulate DNA repair, the cell cycle, and apoptosis, and immune surveillance. Tumors exhibit hypermethylation of the promoters of genes (such as BRCA1, RASSF1A, APC, CDH1 and PTEN) that play key role in tumor progression and resistance to therapy [12]. 5-mC in promoter regions blocks access by transcription factors, favoring binding of methyl-binding proteins leading to chromatin condensation and repression of genes [13]. This results in the abnormal methylation pattern to allow disease to progress and oncogenic transformation.

1.4 Biological Significance of 5-Hydroxymethylcytosine

The discovery of 5-hmC added a new dimension to the world of DNA methylation. Ten-eleven translocation enzymes (TET1, TET2, and TET3) are responsible for the production of 5-hmC through oxidation of 5-mC [14]. This modification is a reversible demethylation intermediate in active demethylation pathways and which is a consistent epigenetic mark with unique epigenetic regulatory characteristics.5-hmC is generally associated with genome differentiation of cells and active transcription [14].Numerous investigations have revealed a significant drop in 5-hmC in many cancer types. Decreased levels of 5-hmC is thought to be linked to high aggressive tumor behavior, poorer prognosis and high metastatic potential [15]. Epigenetic dysregulation and stem cell-like phenotypes have been associated to the reduced TET activity and reduced 5-hmC in TNBC [1]. The low level of 5hmC results in gene expression that make it easier for cells to become malignant. Thus, hydroxymethylation landscape characterization could help to insight into the biology and prognosis of tumors [9].

1.5 Rationale of the Study

Many researchers focused DNA methylation in breast cancer, but there are few studies, that thoroughly examined the methylation and demethylation dynamics in TNBC [4].The co-ordinated epigenetic regulation of 5-mC modification and 5-hmC modification suggests that there is a joint mechanism of epigenetic regulation could lead to the progression of cancer [10].A comprehensive characterization of these epigenetic modifications could be beneficial to understand the heterogeneity of TNBC, as well as the identification of novel biomarkers for prognosis, diagnosis, and treatment targeting [5]. The goal of the current study was to assess the changes of global and gene specific 5-mC and 5-hmC levels and their function in TNBC pathogenesis.

2. RESEARCH GAP

Despite existing evidence supporting the level of abnormal methylation of DNA in TNBC, comprehensive investigations of demethylation and methylation processes have been insufficient so far. The current literature frequently emphasizes the connection between 5-mC and 5-hmC tumor levels prior to therapy and TNBC patient's reaction to NACT. Furthermore, we evaluated the association between these markers. A thorough assessment of 5-hmC and 5-mC is necessary to understand the function of methylation of DNA alterations in TNBC. These analyses might be utilized to determine novel biomarkers and therapeutic targets for precision oncology applications.

3. METHODOLOGY

3.1 Study Design and sample Collection

The study included biopsy tumor samples from forty female patients (age 45-55 years), who had triple-negative breast cancer (TNBC). These forty samples were divided into two groups: Before surgery, 15 patients received neoadjuvant chemotherapy (NACT), whereas 25 patients did not. Patients admitted in surgical oncology department of HSK Hospital and Research Centre, Bagalkot included in the study.

15 patients underwent neoadjuvant chemotherapy, as below –

4 cycles of AC (doxorubicin and cyclophosphamide every 2-3 weeks) followed by 12 cycles of paclitaxel (administered weekly).The Institutional Ethics Certificate was obtained (approval no: BLDE (DU)/IEC/1054/2023-24 dated 13/03/2023) from BLDE (DU) Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, India.

3.2 DNA Isolation

Genomic DNA extraction in tumor samples of TNBC patients was done using the HiGenoMB, Unzipping Genes DNA purification kit (HiMedia Laboratories Pvt Ltd.).Immediately after isolation of DNA the sample was stored at -200C till further analysis [16].

3.3 Quantification Global Methylation and Demethylation Levels

The percentage of global 5-hmC and 5-mC levels were measured by ELISA kit. [17].

3.4 Statistical Analysis

Statistical analysis was done using 'Student t Test' and comparisons involving more than two groups, and SPSS software version 20 was used to evaluate group differences. Pearson's correlation coefficient was used to analyze the link between continuous variables. Scatter plots are used to show correlation findings, and bar graphs with their interquartile ranges and medians are used to show differences.

4. RESULTS

4.1 Comparison of 5-mC and 5-hmC Levels in triple-negative breast cancer patients with NACT & without NACT.

The median levels of 5-mC and 5-hmC in biopsy samples from before surgery subject with NACT accounted for 0.690% and 0.070%, respectively, and were both significantly lower than those observed in tumor samples obtained from subjects without NACT at the time of surgery (2.890% and 0.212 %, respectively, p< 0.0001 for 5-mC, p<0.0001 for 5-hmC;.The two patient groups were statistically Significant.

Table1. Markers of DNA methylation (5-mC) and DNA demethylation (5-hmC) in triple-negative breast cancer Patients with NACT and without NACT.

Methylation and Demethylation Markers	Total Samples (n=40)	Biopsy Samples with NACT (n=15)	Biopsy Samples Without NACT (n=25)	P*
5-mC[%]	2.008 ± 1.102 (0.570 -3.000)	0.685 ± 0.064 (0.570 – 0.785)	2.801 ± 0.471 (0.585 – 3.000)	< 0.0001
5-hmC[%]	0.156 ± 0.068 (0.067 – 0.224)	0.070 ± 0.002 (0.067 – 0.073)	0.207 ± 0.014 (0.185 – 0.224)	< 0.0001

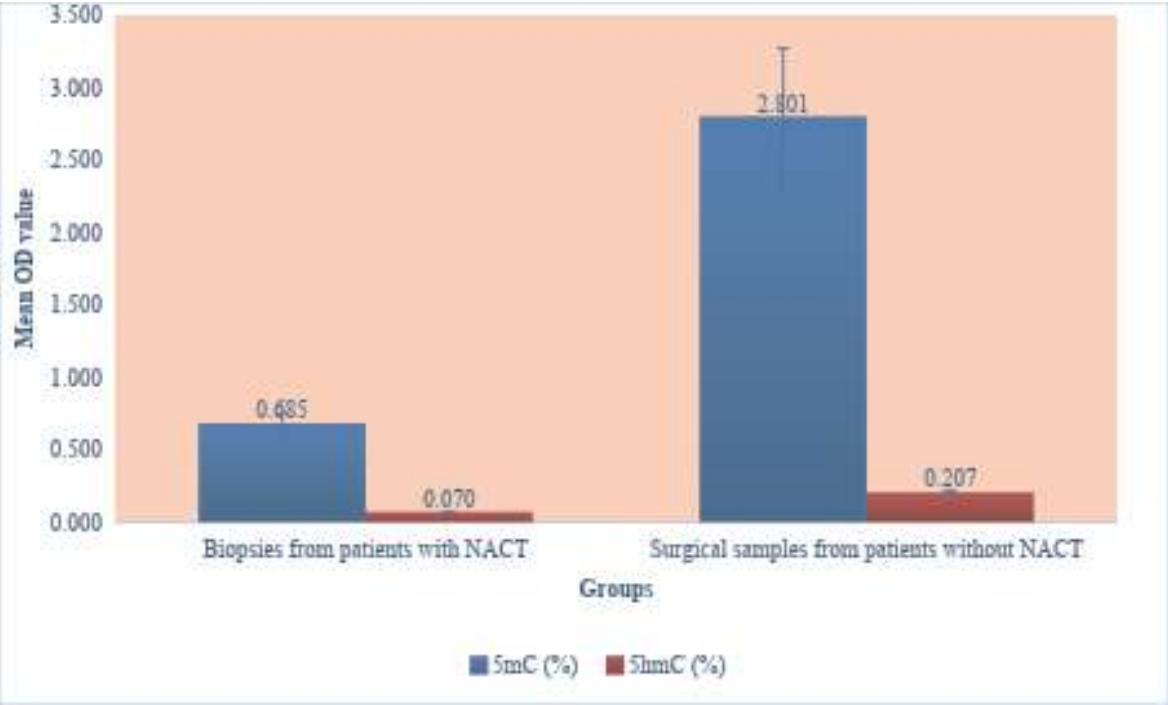
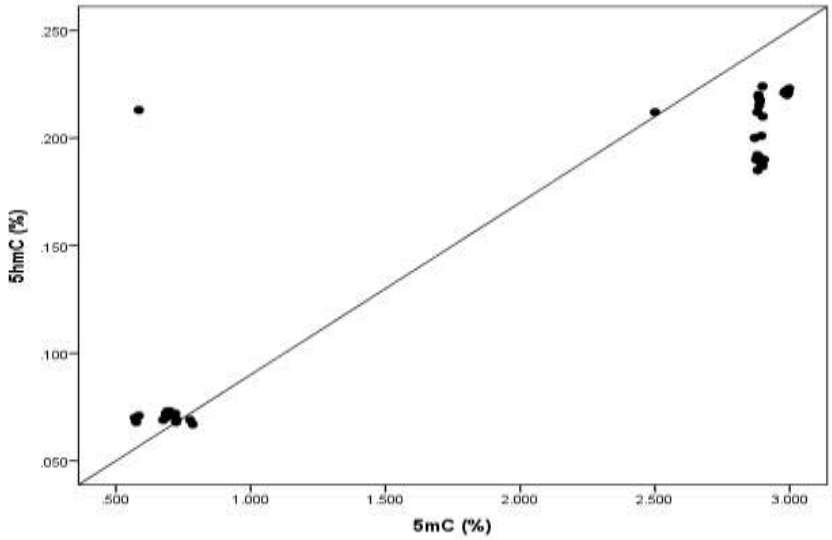
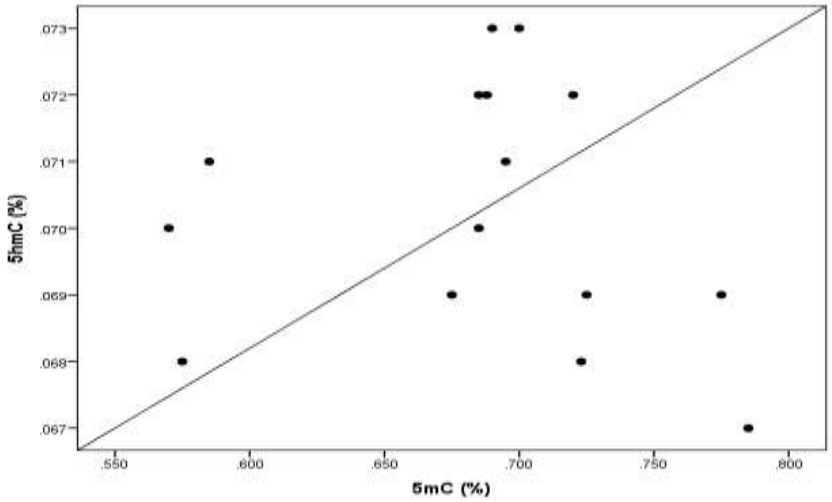


Figure 2 Relationship between markers of DNA methylation (5-mC) and demethylation (5-hmC) in all biopsy samples. [Biopsy samples from all TNBC patients (a), biopsy sample from patients with neoadjuvant chemotherapy (b), and biopsy samples from patients without chemotherapy (c)].

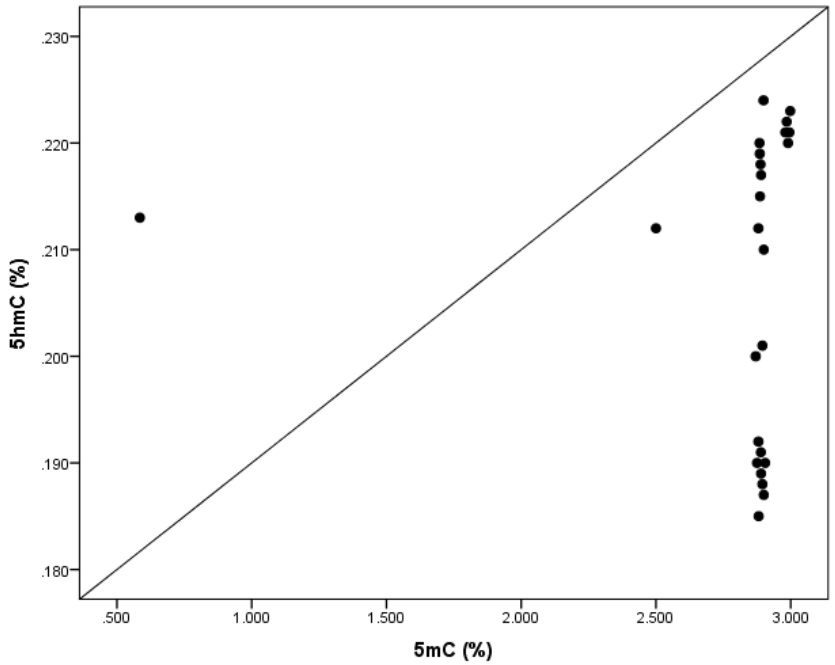
(a) Correlation among DNA methylation of total samples with and without neoadjuvant chemotherapy—(rs = 0.926, p < 0.0001)



b). Correlation between5-mC and 5-hmCin biopsy samples with neoadjuvant chemotherapy– (rs = -0.134, p=0.635)



C). Correlation between5-mC and 5-hmCin biopsy samples without neoadjuvant chemotherapy–(rs = - 0.051, P=810)



5. DISCUSSION

In this study, we aimed to assess the global DNA methylation marker 5-mC and demethylation marker 5-hmC in TNBC patients. These two epigenetic markers are interconnected, as 5-hmC is derived from 5-mC through the action of TET (Ten-Eleven-Translocation) enzymes)[18].In our study we found a positive correlation (rs=0.926, p<0.0001) between these two markers. This equilibrium signifies the functioning of DNA methylation and demethylation processes. We also observed that decreased levels of 5-mC and 5-hmC (0.690% and 0.070%, respectively) in biopsy samples of patients with NACT as compared to without NACT (2.890% and 0.212%, respectively). Some researcher suggested that DNA methylation can be a powerful molecular tool to identify TNB (triple-negative breast cancer) subtypes [19].The study identified that the epigenetic markers are linked to increased levels of differentiation, genome instability, proliferation rate and prognosis of the disease. The report pointed out that alterations in the methylation of DNA suppress the function of genes without altering the sequence of the nucleotides. These epigenetic changes impact the pathways involved in cellular proliferation, apoptosis, invasion and metastasis. Promoter hypomethylation encourages the activation of carcinogenic pathways that aid in malignant transformation, while promoter hypermethylation frequently results in the silence of tumor suppressor genes. Hypermethylation patterns have been linked to a short recurrence-free duration, regardless of whether patients took chemotherapy or not.

Tarhonska et al (2025) has reported that triple-negative breast cancer (TNBC) methylation and demethylation play essential function in controlling biological behavior and treatment response of TNBCs. The study aimed to correlate epigenetic modifications and clinicopathological characteristics with TNBC chemotherapeutic response [4].

We observed that the intensity of the illness and prognosis was strongly correlated with aberrant methylation. Elevated 5-mC and 5-hmC DNA levels were linked to higher proliferation, improved metastatic potential, and a worse response to neoadjuvant chemotherapy (NACT). Concurrently, alterations in the epigenetic process contributed to the ongoing silencing of genes involved in apoptosis, immune regulation, and genomic stability, which implies more aggressive and proliferative features of tumors.

We also observed positive relationship between methylation status and the resistance to chemotherapy. The neoadjuvant Chemotherapeutic drug treatment has different effects on patients with different methylation patterns. Some methylation patterns were linked to treatment sensitivity, while others were linked to the therapeutic resistance. These observations indicate that epigenetic markers are useful in monitoring personalized treatment decisions and clinical outcome. Higher levels of global 5-mC and 5-hmC DNA methylation in TNBC tissues were associated with greater cell proliferation, increased metastatic potential, and a poorer response to neoadjuvant chemotherapy (NACT).

Our findings imply that the combination of DNA methyltransferase inhibitors and neoadjuvant chemotherapy may improve treatment outcomes in patients with triple-negative breast cancer, who have elevated levels of DNA methylation markers. Our results are inconsistent with the researcher that these epigenetic modifications may alter the expression of cancer-related genes, impacting medication susceptibility or resistance. [20-22].

ELISA assays are generally susceptible to significant variability; therefore, this method is suitable for providing rough estimates of gene methylation patterns. Nonetheless, this method is straightforward and fast, making it useful for identifying major genetic changes in the DNA methylome, which potentially indicate a quick screening tool for evaluation of responses to neoadjuvant chemotherapy.

Our findings demonstrated that higher levels of 5-mC and 5-hmC indicators were associated with a poorer response to neoadjuvant treatment in triple-negative breast cancer tissues. These results suggest that hypermethylation could be used as a marker to identify triple-negative breast cancer patients, who are more likely to respond poorly to chemotherapy and have an aggressive course of illness. Understanding these relationships are essential for creating individualized treatment plans and enhancing patient outcomes.

6. LIMITATIONS

Due to relatively low frequency of this specific subtype of breast cancer, we had trouble in obtaining sufficient number of TNBC samples, which limited our sample size. Repeated assessments with and without NACT may reveal the dynamic nature of DNA patterns in response to treatment and its potential as a predictive biomarker for breast cancer survival following neoadjuvant chemotherapy.

7. CONCLUSIONS

The strong positive correlation between 5-mC and 5-hmC levels in TNBC patients' tumor sample suggests that global DNA methylation and demethylation markers may be prognostic of TNBC. Additionally, 5-hmC and 5-mC concentrations in tumor tissues seem to be linked to TNBC patients' response to neoadjuvant chemotherapy, suggesting that these epigenetic changes may be predictive of treatment outcome. Validating these findings in larger cohorts could advance triple-negative breast cancer (TNBC) research and help personalize patient treatment plans.

8. ADDITIONAL INFORMATION

Author Contributions All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Dr. Supriya Aland, Dr. Indira A .Hundekari

9. DISCLOSURES

Human subjects: Bijapur Lingayat District Educational (BLDE) (Deemed to be University) Institutional Ethical Committee issued approval BLDE (DU)/IEC/1054/2023-24 dated 13/03/2023. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects. **Conflicts of interest:** All authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the

submitted work. **Financial relationships:** All authors have

REFERENCES

1. Lehmann U. Epigenetic therapies in triple-negative breast cancer: Concepts, visions, and challenges. Cancers. 2024;16(12):2164.
2. Lin LH, Tran I, Yang Y, Shen G, Miah P, Cotzia P, et al. DNA methylation identifies epigenetic subtypes of triple-negative breast cancers with distinct clinicopathologic and molecular features. Mod Pathol. 2023;36(11):100306.
3. Mohanad M, Hamza HM, Bahnassy AA, Shaarawy S, Ahmed O, El-Mezayen HA, et al. Molecular profiling of breast cancer methylation pattern in triple negative versus non-triple negative breast cancer. Sci Rep. 2025;15(1):6894.
4. Tarhonska K, Wichtowski M, Wow T, Kołacińska-Wow A, Płoszka K, Fendler W, et al. DNA methylation and demethylation in triple-negative breast cancer: associations with clinicopathological characteristics and the chemotherapy response. Biomedicines. 2025;13(3):585.
5. Aine M, Nacer DF, Arbajian E, Veerla S, Karlsson A, Häkkinen J, et al. The DNA methylation landscape of primary triple-negative breast cancer. Nat Commun. 2025;16(1):3041.
6. Ray SK, Mukherjee S. Epigenetic reprogramming and landscape of transcriptomic interactions: Impending therapeutic interference of triple-negative breast cancer in molecular medicine. CurrMol Med. 2022;22(10):835-50.
7. Mandumpala JJ, Baby S, Tom AA, Godugu C, Shankaraiah N. Role of histone demethylases and histone methyltransferases in triple-negative breast cancer: Epigenetic mnemonics. Life Sci. 2022;292:120321.
8. Reddy GD, Brahmaiah V, Kavitha BY, Nethagani J, Palaparthi EC, Lebaka RR, et al. Role of epigenetics variation with focus on DNA methylation in triple-negative breast cancer patients. Arch Med Health Sci. 2023;11(1):96-110.
9. Xie W, Wen Y, Gong S, Long Q, Zou Q. The great potential of DNA methylation in triple-negative breast cancer: From biological basics to clinical application. Biomedicines. 2026;14(1):241.
10. Cai WY, Cai XX, Fei YR, Ye R, Song DM, Hu D, Zhang WW, Xia MF, Yang XX. DNA methylation and immune evasion in triple-negative breast cancer: Challenges and therapeutic opportunities. Front Oncol. 2025;15:1534055.
11. Bagheri M, Lee MK, Muller KE, Miller TW, Pattabiraman DR, Christensen BC. Alteration of DNA methyltransferases by eribulin elicits broad DNA methylation changes with potential therapeutic implications for triple-negative breast cancer. Epigenomics. 2024;16(5):293-308.
12. Umeh-Garcia M, O'Geen H, Simion C, Gephart MH, Segal DJ, Sweeney CA. Aberrant promoter methylation contributes to LRIG1 silencing in basal/triple-negative breast cancer. Br J Cancer. 2022;127(3):436.
13. Zhang H, Gao M, Zhao W, Yu L. The chromatin architectural regulator SND1 mediates metastasis in triple-negative breast cancer by promoting CDH1 gene methylation. Breast Cancer Res. 2023;25(1):129.
14. Ghazimoradi MH, Pakravan K, Khalafizadeh A, Babashah S. TET1 regulates stem cell properties and cell cycle of cancer stem cells in triple-negative breast cancer via DNA demethylation. BiochemPharmacol. 2024;219:115913.
15. Ray SK, Mukherjee S. Epigenetic reprogramming and landscape of transcriptomic interactions: Impending therapeutic interference of triple-negative breast cancer in molecular medicine. CurrMol Med. 2022;22(10):835-50.
16. HiMedia Laboratories Pvt Ltd. HiGenoMB DNA purification kit. Mumbai (India): HiMedia Laboratories Pvt Ltd.
17. Epigentek. MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric). Base Catalog No. P-1030. Farmingdale (NY): Epigentek.
18. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324(5929):930-5.
19. International Agency for Research on Cancer. Cancer Today [Internet]. Lyon: Global Cancer Observatory; [cited 2025 Feb 10]. Available from: https://gco.iarc.who.int/today/en
20. Mirza S, Sharma G, Pandya P, Ralhan R. Demethylating agent 5-aza-2-deoxycytidine enhances susceptibility of breast cancer cells to anticancer agents. Mol Cell Biochem. 2010;342(1-2):101-9.
21. Sandhu R, Rivenbark AG, Coleman WB. Enhancement of chemotherapeutic efficacy in hypermethylator breast cancer cells through targeted and pharmacologic inhibition of DNMT3b. Breast Cancer Res Treat. 2012;131(2):385-99.
22. Buocikova V, Longhin EM, Pilalis E, Mastrokalou C, Miklikova S, Cihova M, et al. Decitabine potentiates efficacy of doxorubicin in preclinical trastuzumab-resistant HER2-positive breast cancer models. Biomed Pharmacother. 2022; 147:112662.