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DNA Methylation in Human Breast Cancer Cell Lines Adapted to High Nitric Oxide

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Abstract. *Background: Nitric oxide (NO) exposure has been suggested to cause alterations in DNA methylation in breast cancer. We investigated the effect of NO on DNA methylation of promoters in cell lines of breast cancer. Material and Methods: The methylation status of the promoters of breast cancer 1 (BRCA1), deleted in colon cancer (DCC), Ras-association domain family 1A (RASSF1A), O^6-methylguanine-DNA methyltransferase (MGMT), and secreted frizzled related protein 1 (SFRP1) were analyzed in the parental and high nitric oxide-adapted cell lines of breast cancer using Illumina MiSequencing. Results: Methylation of RASSF1A promoter in BT-20-HNO (74.7%) was significantly higher than that in BT-20 cells (72%) ($p<0.05$), whereas in MCF-7-HNO cells, methylation of MGMT promoter was found to have significantly decreased as compared to its parental cell line (45.1% versus 50.1%; $p<0.0001$). Promoter methylation of SFRP and DCC was elevated in T-47D-HNO relative to its parent cell line ($p<0.05$). Conclusion: Similarly to the double-edged effects of NO on tumorigenesis, its epigenetic effects through DNA methylation are diverse and contradictory in breast cancer.*

Breast cancer is the most prevalent malignancy and second most common cause of cancer deaths among females (1-3). It affects approximately 1.5 million women worldwide each year (1). Patients with breast cancer with morphologically identical tumors exhibit different clinical courses. This may be the result of variations in the tumor microenvironment. It has been proposed that the tumor microenvironment has a crucial role in tumor initiation as well as progression and metastasis. The free radical nitric oxide ($^{\bullet}$NO) is believed to be a factor at play in the tumor microenvironment (4-7). It has multiple effects on tumor biology. Depending on the dose and duration of exposure, NO exhibits its biological effects by either inhibiting or stimulating cell proliferation, migration and apoptosis (8). NO is reported to cause harm in the adipogenic environment of the breast, and thus initiates and promotes tumor development. Reactive oxygen species (ROS) and NO originating in the tumor microenvironment produce oxidative stress and inflammatory factors. Therefore, they change the microenvironment of the breast and create an environment for the transformation of breast cancer cells (9). NO is produced by nitric oxide synthase (NOS) which has three isoforms, neuronal, endothelial (eNOS) and inducible (iNOS) (5). Increased expression of NOS has been identified in human tumors including breast cancer (4, 5, 8, 9). iNOS is a proinflammatory enzyme involved in chronic inflammation. It has been shown that in patients with triple-negative breast tumors, iNOS expression is associated with worse prognosis and poor survival (5, 8, 9).

Epigenetic mechanisms have appeared as essential players in the development and progression of breast cancer (1). DNA methylation is one of these mechanisms that regulate the differential expression of genes (10-12). It occurs at cytosines contained within cytosine-phosphate-guanine (CpG) dinucleotides. They are converted to 5-methylcytosine by incorporation of a methyl (CH_3) group to the fifth carbon of the pyrimidine ring of cytosine. DNA methyltransferases (DNMTs) are the main enzymes which regulate DNA methylation (1, 10). Abnormal DNA methylation of promoter CpG islands is often correlated with reduced transcriptional activity in cancer (12, 13).

Epigenetic silencing *via* promoter hypermethylation of more than 100 genes has been reported in breast cancer. These silenced genes are involved in cell-cycle regulation, DNA repair, tissue invasion, apoptosis and metastasis (1). Most of these mechanisms are also modulated by NO as

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explained above. This suggests that NO exposure might cause alterations in DNA methylation. NO has been already reported to be an epigenetic factor due to its impact on DNA methylation, microRNA and histone modification in normal as well as tumor tissues (14, 15). However, to our knowledge, the epigenetic effects of NO in terms of DNA methylation of promoters have never been investigated in breast cancer. To fully understand the influence of NO on DNA methylation in breast cancer, we utilized a model cell line system which was previously designed by our laboratory utilizing some of the breast cancer cell lines that were adapted to NO (16-23). These cells were progressively adapted to high concentrations of NO over a period of 2 months (4). At the end of the adaptation process, two significant results were obtained. Firstly, the untreated cell lines which we called 'parental' cells adapted to the high NO (HNO) level. Secondly, HNO-adapted cell lines expanded more quickly and aggressively than each parental cell line. It was observed that HNO-adapted cells continued to grow in the same way even when they were later grown without NO treatment (4, 5).

In this study, we investigated and compared the methylation status of promoter regions of breast cancer 1 *(BRCA1)*, deleted in colon cancer (*DCC*), Ras-association domain family 1A (*RASSF1A*), O^6-methylguanine-DNA methyltransferase (*MGMT*), and secreted frizzled related protein 1 (*SFRP1*) tumor-suppressor genes between parental breast cancer cell lines and their HNO-adapted derivatives.6

Materials and Methods

Cell lines and cell adaptation. Three human breast adenocarcinoma cell lines were utilized for this study: BT-20, MCF-7, and T-47D. These cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). T-47D was cultured in RPMI-1640 media. MCF-7 and BT-20 were each cultured in Minimum Essential Medium (MEM). How these three cell lineswere adapted to grow in comparatively high levels of NO has been reported (4). Briefly, the cell adaptation process was conducted over several months by exposing the parentaI cell lines to an NO donor, (Z)-1-[2-(2-aminoethyl)-N-(2-amminoethyl) amino] diazen-1-ium-1,2-diolate (Sigma Life Sciences, St. Louis, MO, USA), at an initial concentration of 50 μM. The concentration was raised in increments of 25 μM up to a point which was lethal to the parentaI cell line (a concentration of 600 μM) every few days. The newly adapted HNO cell lines were designated as BT-20-HNO, MCF-7-HNO, and T-47D-HNO, respectively. Each media type was supplemented with 10% fetal calf serum (which was inactivated at 56˚C for 30 min prior to use), 100 μg/ml streptomycin, 2 mM L-glutamine, 2.5 μg/ml amphotericin B solution, and 100 U/ml penicillin. In addition, MEM was supplemented with 1 mM sodium pyruvate and 100 mM nonessential amino acids (CellGro, Inc. Manassas, VA, USA). All cell lines were maintained in a humidified incubator at 37˚C and a concentration CO_2 of 5%.

Extraction and sodium bisulfite modification of genomic DNA. Genomic DNA was isolated from cultured human breast tumor cell lines by Qiagen Blood and Cell Culture DNA kit (Qiagen, Inc., Valencia, VA, USA) and stored at −20˚C before use. Isolated genomic DNA samples were treated with bisulfite deamination reaction using Qiagen EpiTect Bisulfite kit (Qiagen, Inc., Valencia, VA, USA) based on the manufacturer's instructions. Briefly, 500 ng of DNA was used from each sample. The required amount of DNA was mixed with 85 μl of bisulfite mix solution and 35 μl of DNA preservation buffer in 200 μl polymerase chain reaction (PCR) tubes. Samples were then incubated in a thermal cycler device (BioRad, Hercules, CA, USA) for 5 hours and in changing temperatures (95˚C for 5 min, 60˚C for 25 min, 95˚C for 5 min, 60˚C for 85 min, 95˚C for 5 min, and 60˚C for 175 min, respectively). Samples were transferred to the Epitect spin columns (Qiagen, Inc., Valencia, VA, USA) after incubation, and desulfonation and washing buffers were added and centrifuged accordingly. Finally, bisulfite-treated DNA samples were purified in 20 μl of elution buffer. One microliter of bisulfite-converted genomic DNA solution was used in subsequent PCR reactions.

PCR amplification and sequencing of BRCA1, DCC, RASSF1A, MGMT, SFRP1. PCR reactions were performed using bisulfite-converted genomic DNA using primers listed in Table I. PCR reactions were carried out *via* Qiagen HotStarTaq DNA polymerase and supplied 1X PCR buffer supplemented with 0.1 mmol/l dNTPs, 2.5 mmol/l $MgCl_2$, and 0.5 μmol/l each of forward and reverse primers and bisulfite-converted genomic DNA. The thermal cycler (BioRad, USA) was set up for initial activation step for 15 min at 95˚C, and followed by 45 cycling steps of 94˚C 30 s, optimized primer specific annealing temperature for 30 s, and 72˚C 30 s. The PCR reaction was complete after the final elongation step applied at 72˚C for 10 min. Following amplification, the PCR products were loaded onto 10% polyacrylamide gels and visualized using ethidium bromide. Human placental genomic DNA (Biochain Institute, Hayward, CA, USA) was used as positive and negative control. This genomic DNA was either methylated *in vitro* with *SssI* methylase (NEB, Ipswich, MA, USA) or not methylated prior to bisulfite conversion as outlined previously (24). The PCR reactions were then analyzed by sequencing (control DNA was used for validation as described above).

Samples were then prepared for sequencing by ligation of barcoded sequencing adapters using a PrepX kit implemented on an Apollo 324 robotic system (IntegenX Inc., Pleasanton, CA, USA). Barcoded adapters were NEXTflex 6nt barcodes (Bio Scientific, Phoenix, AZ, USA). Sequencing was conducted on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) employing V3 chemistry (600 cycles). Library preparation and pooling of samples was performed by the University of Illinois at Chicago Sequencing Core Facility. Sequencing was carried out at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign.

Raw sequence data were processed within the software package CLC genomics workbench (Qiagen, Redwood City, CA, USA). For each sample, from 450,303 to 827,542 clusters were acquired. Quality trimming (Q20, no ambiguous nucleotides allowed) was performed on all samples, and trimmed reads were mapped against reference 'converted' sequences for each gene (*i.e.* C positions were converted to T). Subsequently, variant calling was performed using the default CLC variant caller. Variant calling tables for each sample were exported, and for each nucleotide position, the number of sequences generating each base were counted. Wilcoxon matched-pairs signed-rank test was employed to compare the differences in

Table I. *Sequencing primers (amplification and sequencing).*

Gene name	Encoded protein	Primer sequence (5' → 3')	Orientation	Annealing temp (°C)	Thermo-cycles	Product size (bp)
BRCA1	Breast cancer 1	GGGATAAGTGGTAAGAGTTAATTTTTTT	F	58	45	189
		CACCCCTAACTAACCCAAACTACT	R			
		GTGGTAAGAGTTAATTTTTTT	Seq*			
DCC	Deleted in colon cancer	GAAATTAATAGGGAATGGTATATTAAT	F	58	45	273
		TCTCTCTATCTCTAACCAAAAAAAA	R			
		TAGGGAATGGTATATTAAT	Seq*			
RASSF1A	Ras-association domain family 1A	GTAGTTTAATGAGTTTAGGTTTTTT	F	59	45	188
		CTACACCCAAATTTCCATTAC	R			
		TAATGAGTTTAGGTTTTTT	Seq*			
MGMT	O^6-Methylguanine-DNA methyltransferase	GGTTTGGGGGTTTTTGATTAG	F	57.5	45	198
		CCTTTTCCTATCACAAAAATAATCC	R			
		GGGGGTTTTTGATTAG	Seq*			
SFRP1	Secreted frizzled related protein 1	GGGGAATTTGTTATATTTAAGTATTT	F	58	45	192
		ATACCCCTACTCAACAAAAACTACC	R			
		TTGTTATATTTAAGTATTT	Seq*			

Seq*: Sequencing primer.

methylation between the parental and HNO-adapted cell lines, and Graphpad prism v6 (GraphPad Software, San Diego, CA, USA) program was used to conduct the statistical analysis.

Results

Comparison of promoter CpG methylation levels of BRCA1, DCC, RASSF1A, MGMT and SFRP1 between the parental and HNO-adapted cell lines by Illumina Mi-Sequencing. The tumor-suppressor genes we studied (*BRCA1, DCC, RASSF1A, MGMT* and *SFRP1*) have been shown to be epigenetically silenced to various extents by promoter DNA hypermethylation in breast cancer (25-30).

To perform PCR and sequencing analysis, we designed primers using an on-line primer design program (www.methprimer.org), which is publically available. The primer sets of PCR and MiSeq analysis were designed to determine DNA methylation levels near to the transcriptional start site of the tumor-suppressor genes because this region is most likely to be exposed to dense methylation during epigenetic silencing (31). By utilizing unmethylated and methylated human placental genomic DNA as negative and positive controls, respectively, we validated our results of sequencing analysis for the five genes *(BRCA1, DCC, RASSF1A, MGMT and SFRP1*) studied in this report (Figure 1). Based on the sequencing protocol, the libraries from different samples were quantified and pooled together. After sequencing, the Illumina reads were post-processed and aligned to the human reference regions.

For all five genes, we detected promoter hypermethylation to various extents in the parental cell lines (BT-20, MCF-7, and T-47D) which was consistent with previous reports.

We first examined the sequencing results of *BRCA1* by Illumina reads. The primers designed to amplify the *BRCA1* gene sequence yielded a 189-bp amplicon and included 10 CpG dinucleotides. The results indicated that methylation of *BRCA1* did not significantly change in response to NO treatment in any of the cell lines (Figure 1).

A 188-bp segment from the CpG island of the RASSF1A promoter was amplified by PCR and used for MiSeq. Illumina MiSeq assay for the *RASSF1A* gene scores methylation of 16 CpG dinucleotides within the amplified region of the CpG island within the 5' end of the gene. Based on the sequencing results, we observed that methylation level of *RASSF1A* in BT-20-HNO (74.7%) cell was significantly ($p<0.05$) higher than that of the parental line (72%) but there was no difference between the other parentaI and HNO-adapted cell lines.

MGMT was another tumor-suppressor gene whose promoter methylation status (198-bp segment including 21 CpG) which was analyzed by sequencing following PCR amplification. The MCF-7-HNO cell line (45.1%) was found to have a significantly ($p<0.0001$) reduced methylation of *MGMT* as compared to its parental cell line (50.1%). However, HNO-adapted BT-20 and T-47D cell lines had similar methylation levels when compared to their corresponding parental lines.

SFRP (192-bp segment with eighteen CpG) sequencing results demonstrated that *SFRP* methylation was elevated in the T-47D-HNO (56.2%) cell line relative to its parental cell line (55.0%) ($p<0.05$), while no significant change was observed between other cell lines.

Sequence analysis of *DCC* tumor-suppressor gene (273-bp segment amplified with twenty CpG prior to MiSeq)

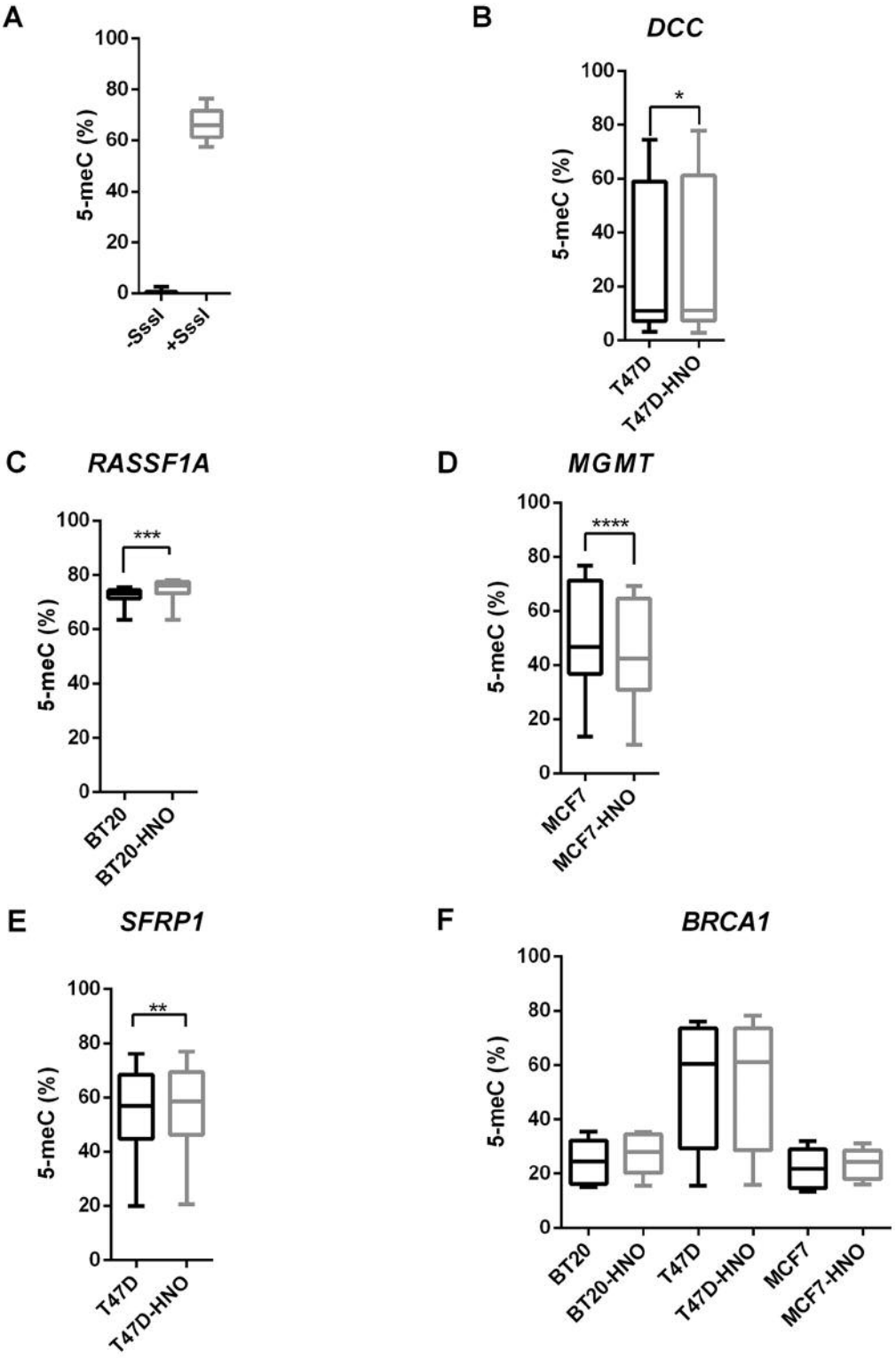

Figure 1. *A: DNA methylation in control samples of placental DNA treated with SssI (+) and untreated (–). DNA methylation of promoters for genes in parentaI and high nitric oxide (HN0)-adapted cell lines: Deleted in colon cancer (DCC) in T47-D cells (B); Ras-association domain family 1A (RASSF1A) in BT20 cells (C); O^6-methylguanine-DNA methyltransferase (MGMT) in MCF7 cells (D); secreted frizzled related protein 1 (SFRP1) in T47-D cells (E); and breast cancer 1 (BRCA1) in BT-20, MCF-7 and T-47D cells (F). Data are presented as mean±standard deviation. Significantly different at: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.*

showed that the level of methylation in T-47D-HNO (30.7%) cells was significantly ($p<0.05$) higher than in the parental cel line (29.7%). No difference was detected between the parental and HNO cell line pairs for BT-20, and MCF-7 (Figure 1).

Discussion

NO is an unstable, reactive, free radical, and also an essential signal molecule in sustaining cellular homeostasis. It plays a critical role in physiological as well as pathological processes such as inflammation and cancer (32, 33). It has been shown that NO levels are higher in breast cancer as compared to that in benign breast epithelium (8, 9). Our previous studies characterized some molecular and cellular alterations induced by high NO concentration in human breast cancer using a model cell line system which was developed by our laboratory.

In order to make the role of NO clear epigenetically, we detected the level of methylation of a panel of five tumor-suppressor genes in the same model system of breast cancer cell lines in the current study. The tumor-suppressor genes *BRCA1, DCC, RASSF1A, MGMT* and *SFRP1* were chosen since they have been shown to be subjected to epigenetic silencing by promoter hypermethylation in primary breast cancer (25-30). We used Illumina MiSequncing to quantify methylation of these genes in parental and HNO-adapted breast cancer cell lines and observed diverse results in response to NO treatment. Placental gDNA sequencing acted as our negative and positive controls (*–SssI* and *+SssI*) (Figure 1).

BRCA1 is a classic tumor-suppressor gene and is associated with the regulation of gene transcription, DNA repair, apoptosis, cell-cycle checkpoint control and remodeling of chromosomes (34, 35). Inherited germline mutations in *BRCA1* result in the formation of aggressive breast tumors. In addition, it has been reported that DNA methylation was the major cause of *BRCA1* gene silencing, ranging from 10-30% in sporadic breast cancer (36-39). Our findings on *BRCA1* promoter methylation of BT-20, MCF-7, and T-47D were consistent with previous studies. However, treatment of these cell lines with NO did not significantly change the level of methylation of *BRCA1* gene promoter (Figure 1).

RASSF1A is involved in microtubule stability, cell-cycle progression and apoptosis (40). Promoter hypermethylation of *RASSF1A* has been observed in breast cancer in recent studies (41-44). We found a significantly elevated level of methylation of RASSF1A promoter only in the BT-20-derived cell line out of four HNO-adapted breast cancer cell lines. In penwork from our laboratory, it was observed that iNOS and eNOS expression was significantly up-regulated in BT-20 HNO cells compared to the parental cell line (5), which was in agreement with the literature reporting the link of high NOS expression and NO levels with breast cancer (8, 9).

We subsequently demonstrated the relationship between promoter methylation levels of *DCC* and *SFRP1* genes in the T47D cell line and high NO concentrations. *SFRP1* is a member of the frizzle protein family and negatively regulates the Wnt signalling pathway (45-47). *DCC* is involved in cell progression, migration and adhesion (48). Both *SFRP1* (30) and *DCC* (29) have been shown to be epigenetically silenced by DNA hypermethylation in breast cancer. In the current study, these genes had significantly higher levels of methylation in T47D-HNO cells than in the parental cell line. Unlike up-regulation of iNOS and eNOS expression in BT-20-HNO cells as stated above, expression of both isoforms in T-47D-HNO cells was reduced compared to the parental cell line (5).

Another tumor-suppressor gene we studied was *MGMT*, which functions in DNA repair (49), and whose aberrant promoter hypermethylation has been shown in one-third of breast cancer cases (28). Contrary to the elevatin of methylation of *RASSF1A, SFRP1* and *DCC* promoter in at least one HNO-adapted breast cancer cell line, we observed that methylation of *MGMT* promoter was significantly reduced in MCF-7-HNO cells compared to the parentaI cell line. Western blot analysis demonstrated that iNOS expression was also lower in MCF-7-HNO cells than the parentaI cells, while eNOS expression was higher (5).

Taken together, our findings suggest that much like the double-edged effects of NO on tumorigenesis, its epigenetic impacts through DNA methylation are diverse and contradictory. To the best of our knowledge, there are no reports about the effects of NO on promoter DNA methylation in breast cancer. However, studies performed in various types of cancer and diseases in order to understand the epigenetic role of NO have yielded conflicting results. While some reported that NO induced a global decrease in methylcytosine, others found CpG hypermethylation and subsequent transcriptional silencing. For example, Hmadcha *et al*. observed that direct application of NO donors to several cell types induced promoter methylation of fragile X mental retardation gene (*FMR1*), which led to the suppression of the expression of this gene (50). Another study using a murine squamous cell carcinoma model demonstrated that NO overexpression resulted in a global decrease in DNA methyltransferase 1 (DNMT1) and DNMT3a activity, and in 5-methylcytosine level (51).

Huang *et al*. showed that treatment of gastric cancer cells with *Helicobacler pylori* stimulated an increase in NO level, DNMT activity and DNA methylation (52). In addition, Huang *et al*. (52) and Katayama *et al*. (53) found that NO induced promoter DNA methylation of E-cadherin and Runt-related transcription factor 3 (RUNX3) respectively, in gastric cancer.

The findings outlined above suggest that it is difficult to establish a direct connection between NO and DNA methylation. New approaches toward understanding the influence of NO on epigenetic mechanisms, including the interactions of NO with iron and its effect in the modulation of iron homeostasis, have been proposed by some researchers. Cellular iron is essential for the activity of a wide range of diverse enzymes (54). A number of epigenetic-regulatory enzymes are iron-dependent, such as histone demethylases, which NO inhibits by binding to the catalytic iron (55, 56). Another iron-dependent epigenetic regulatory enzyme is ten-eleven translocation (TET) which catalyzes DNA demethylation in a process that removes or modifies the methyl group from 5-methylcytosine. TET enzyme uses iron, α-ketoglutarate and oxygen to convert 5-methylcytosine to 5-hydroxymethylcytosine, which is associated with active gene transcription (55-57). It has been proposed that inhibition of TET activity by NO might alter the expression of specific genes due to the accumulation of 5-methylcytosine in the genome (55, 56). Thus, NO might result in epigenetic silencing of spesific tumor-suppressor genes by inducing DNA hypermethylation.

NO has three sources in the body, namely dietary, pharmacological and endogenous enzymatic synthesis. However, which sources of NO are more susceptible to epigenetic regulation is unknown (10). For this reason, future investigations to reveal epigenetic effects of NO should take into consideration this point.

In conclusion, NO-induced epigenetic modifications may positively or negatively regulate gene expression. As most epigenetic modifications are reversible, targeting NO might restore epigenetic modifications in tumorigenesis, hence proving to be an efficient therapeutic strategy. Further studies are needed to completely elucidate NO-induced epigenetic mechanisms and develop new epigenetic treatment options in breast cancer.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

Authors' Contributions

BD and JAR designed the project and experiments. BD performed cell adaptation process, DNA extraction, bisulfite modification, PCR and sequencing analysis. BY performed data analysis and statisitcal analysis. BD and JAR wrote/revised the article. All Authors read and approved the final article.

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