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Epigenetic Regulation of *P21*, *RASSF1*, *PTEN* and *P53* in Human Breast Cancer Cells Using Different Chemotherapeutic Drugs

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Abstract: Breast cancer is considered one of the most heterogeneous diseases that might be difficult to characterize and then to treat. Several research groups worldwide have explored different genetic and epigenetic approaches that might help. In the present study, different kinds of chemotherapeutic drugs have been applied to breast cancer cells (MCF-7) to assess their role in changing the methylome of these malignant cells. Global methylation was quantified in treated and untreated cells and the results obtained indicated that the drugs/drug combinations applied (temozolomide, carboplatin, sodium phenylbutyrate, cyclophosphamide, erlotinib, procaine, vorinostat and combinations) have a tremendous effect on the methylation landscape of the cells. Real time PCR was employed to assess the level of expression of different tumor suppressor genes; *RASSF1*, *PTEN*, *P21* and *P53*. The results indicated that applying different drugs/drug combinations has affected the level of gene expression of the above mentioned genes. All drugs/drug combinations were incubated for 4 and 8 days with the cells. The four-day and the eight-day incubation have resulted in down regulation of *P53*, *PTEN* and *P12* while an up regulation of *RASSF1* gene was obtained. This might indicate that the time of incubation has no effect on the regulation of the expression of these genes. However, this study needs more conformational investigation to elucidate the mode of action of the used drugs in epigenetically regulating these genes.

Key words: Epigenetics · Tumor suppressor genes · Oncogenes · Chemotherapeutic drugs · MCF-7 · Real time PCR

INTRODUCTION

Cancer, a genetic disease that is caused when normal cellular functions are disrupted by mutations arising in DNA. These changes occur at the level of single cells and then propagated into subpopulations as cells divide and pass mutations through cell lineages [1].

Breast cancer is a malignancy arising from the epithelial tissues that lining the milk gland or ducts of the breast (ductal epithelium) [2]. Breast cancers have been classified into four subtypes; luminal A, luminal B, HER-2 positive and triple-negative according to the status of hormonal receptors, Ki-67 and human epidermal growth factor receptor-2 (HER-2) expression [3].

Epigenetics refer to all heritable changes to the regulation of gene expression without causing any change in DNA sequence [4]. The major epigenetic alterations that are known to effect on the gene regulation as follows; DNA methylation involves the addition of methyl groups to the 5' carbon at cytosine residues in promoter-rich cytosine phosphate guanine (CpG) islands [5]. There exist four DNMTs; DNMT 1, 2 and 3a and 3b of which DNMT 1 and DNMT 3 have been observed to play a pivotal role in DNA methylation [6]. Histone acetylation caused by the addition of acetyl groups that makes a disturbance in gene transcription, DNA replication, DNA repair or chromatin condensation [7]. Chromatin remodeling proteins that indirectly affect gene transcription by altering the chromatin structures [8].

There area variety of genetic and epigenetic changes in tumor suppressor gene and oncogenes have been implicated in the development of breast cancer in human: transcriptional silencing of tumor suppressor genes which including genes involved in DNA repair, detoxification,

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apoptosis, cell cycle, cell proliferation, metastasis and angiogenesis are observed more often in breast cancer patients compared with cancer-free controls [9], promoter hypermethylation may also suppress the transcription of tumor suppressor genes, while hypomethylation of CpG islands can result in genome instability, reactivation of transposons and upregulation of proto-oncogenes [10]. The present study focused on elucidating the role of DNA methylation in the regulation of some breast cancer-related genes such as *P53, PTEN, RASSF1A* and *P21*.

Vorinostat works as HDAC inhibitors and is used in cancer therapy by promoting growth arrest, differentiation and apoptosis of cancer cells with minimal effects on normal tissue [11], while cyclophosphamide considered one from Alkylating agents that acts by inhibiting the transcription of DNA into RNA and thereby stopping the protein synthesis [12], temozolomide also included in the alkylating agents but it interferes with the development of cancer cells by slowing down their growth and spread in the body [13], procaine related to DNMT inhibitors category and has an inhibitory effect on growth, causing mitotic arrest besides this, it restores the expression of tumor suppressor genes epigenetically silenced [14], erlotinib was also approved for the first-line treatment of patients whose tumors harbor activating mutations of the epidermal growth factor receptor gene (*EGFR*) due to The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) activity [15], carboplatin is one of the main platinum-based drug used as an antitumor drug, the main target of carboplatin is DNA, to which it binds efficiently, thereby inhibiting replication and transcription and inducing cell death. The nature of these DNA adducts affects a number of transduction pathways and triggers apoptosis or necrosis in tumor cells [16], the final type of chemotherapeutic drug that has used in this study called sodium phenylbutyrate which is related to histone deacetylase inhibitor category, the compound is capable of modulating the structure of chromatin and contributes to the regulation of multiple cell cycle and apoptosis-related genes [17].

MATERIALS AND METHODS

Cell Line Maintenance: The MCF-7 cells were maintained in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic mix. Cells were grown in humidified conditions with 5% CO_2 at 37°C.

Cell Viability Test: Trypan blue test was performed initially to assess the number of viable cells prior subjecting the cells to any treatment. Briefly, 10µL of cell suspension was mixed with 10µL of Trypan blue and the mix was applied to the hemocytometer slide and then visualized under inverted microscope.

Chemotherapeutic Drugs: Erlotinib, temozolomide, cyclophosphamide, procaine, carboplatin, vorinostat and sodium Phenylbutyrate were purchased from Santa Cruz Biotechnology (USA). A stock of 5mg/mL of each drug was prepared. Each drug was dissolved in its appropriate solvent and kept in 4°C until being used.

Drug Application: Seven different drugs/drug combinations were applied. MCF-7 (1 x 10^6 cell/well) was subdivided into four 12-well plates. Combinations were mixed separately and added to the wells containing the MCF-7 cells. Two plates were loaded with the specified drugs (see plate format, Table 1 and 2) and served as the first harvest, where the cells have been harvested after 4 days. The other two plates were loaded with the same drugs/drug combinations and served as the second harvest where the cells have been harvested after 8 days.

DNA Extraction: Genomic DNA was extracted from treated and untreated cells for the downstream analysis *i.e.* methylation quantification in the malignant cells after being treated with the drugs. Extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the kit's instructions.

DNA Integrity: The integrity and purity of DNA samples was checked by 1% (w/v) agarose gel electrophoresis. Gels were subjected to 120 V for 10 minutes and followed by 60 minutes at 100 V. Gels were photographed after being stained with Ethidium Bromide.

DNA Quantification: DNA input were determined for the subsequent step, Invitrogen™ Qubit 2.0 Fluorometer with the Qubit dsDNA BR assay were used to determine double-stranded DNA concentration and purity. The kit's instructions were followed.

Global DNA Methylation Quantification: Global methylation in the treated and untreated cells was measured using Global DNA Methylation kit (Cell Biolabs, USA) with minor modifications. Briefly, the extracted genomic DNA was applied to the assay wells after making the denaturation step. 5-MedCyd DNA conjugate plate was rehydrated. Unknown sample or pre-prepared 5-MedCyd standard was added to the wells. Anti-5MedCyd primary and secondary antibodies were added to the plate. The flouro assay solution was added after washing

Table 1: Plate I represent the combinations and concentrations used in the present study

C	15µL P	15µL Cy	12µL T
3 mL cell susp.	2985µLcell susp.	2985µL cell susp.	2988µLcell susp.
22µL CA	15µLV	11µL S	23µL E
2978µLcell susp.	2985µLcell susp.	2989µLcell susp.	2977µLcell susp.
15µL P	15µL P	15µL P	15µL Cy
15µLV	11µL S	23µL E	15µLV
2970µLcell susp.	2974µLcell susp.	2962µLcell susp.	2970µLcell susp.

C: Control, Cy: Cyclophosphamide, So: Sodium Phenylbutyrate, E: Erlotinib, P: Procaine, V: Vorinostat, CA: carboplatin and T: Temozolomide.

Table 2: Plate II represent the combinations and concentrations used in the present study

15µL Cy	15µL Cy	12µL T	12µL T
11µL S	23µL E	15µLV	11µL S
2974µLcell susp.	2962µLcell susp.	2973µLcell susp.	2977µLcell susp.
12µL T	22µL CA	22µL CA	22µL CA
23µL E	15µLV	11µL S	23µLE
2966µLcell susp.	2963µLcell susp.	2976µLcell susp.	2955µLcell susp.
C	C	C	Blank
3 mL cell susp.	3 mL cells	3 mL cells	

C: Control, Cy: Cyclophosphamide, So: Sodium Phenylbutyrate, E: Erlotinib, P: Procaine, V: Vorinostat, CA: carboplatin and T: Temozolomide.

and then the signals were measured immediately at 450 nm using plate reader. A standard curve was generated to calculate the concentrations of 5-MedCyd in the treated and untreated samples (Figure 1). There were some missing samples during the collection of cells after treatment; carboplatin and the combination of cyclophosphamide with vorinostat.

RNA Extraction: RNA extraction from treated and untreated cells was carried for the subsequent analysis *i.e.* assesses the levels of tumor suppressor genes and oncogenes expression in all samples before and after treatment with chemotherapeutic drugs. Extraction was performed using QIAamp RNA Blood Mini Kit (Qiagen, Germany) the kit instructions were followed.

Complementary DNA (cDNA) Synthesis: cDNA was synthesized from total RNA samples using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and the procedure was carried out according to manufacturer's instructions.

Quantitative Real time PCR (qRT-PCR): The generated cDNA was subjected to Real Time PCR against *P53, P21, PTEN* and *RASSF1A* specific primers (10 pmol each). Gene expression was calculated according to the cycle threshold in both treated and untreated cells. Total reaction volume was 20 µL containing 10 µL of SYBR Green Master Mix. Thermal cycler program was 95°C for preheating step for 10 min, 40 cycles of 15 seconds at 95°C and 60°C for 1 minute for both annealing and extension steps. *GAPDH* was used as internal control.

RESULTS AND DISCUSSION

The main purpose of the present study was to identify the changes of the methylation patterns of *P21*, *P53*, *PTEN* and *RASSF1* tumor suppressor genes in the MCF-7 breast cancer cells after being treated with different chemotherapeutic drugs.

Quantification of DNA Methylation: In the present study, after DNA quantity and quality were checked, the global methylation level in all treated and untreated MCF-7 cells was measured. In this assay, 5-MedCyd concentration was measured as indicator for the global hypo/hypermethylation as a result of the treatments. A standard curve was generated before applying the samples (Fig. 1). Global DNA Methylation readings obtained after the treatment with chemotherapeutic drug(s) were illustrated in (Fig. 2). The obtained data shows that the MCF-7 responded differently to the chemotherapeutic drugs/drug combinations applied. Temozolomide applied for eight days solely or in combination with erlotinib or sodium phenylbutyrate resulted in hypermethylation of the global genome compared to control cells [18-20]. Erlotinib and cyclophosphamide applied solely has increased also the level of global methylation [21, 22]. Meanwhile, a hypermethylation pattern was obtained when sodium phenylbutyrate was applied in combination with cyclophosphamide and carboplatin [23]. These profiles might be attributed to the action of the chemotherapeutic drugs on DNMT and/or HDAC [24].

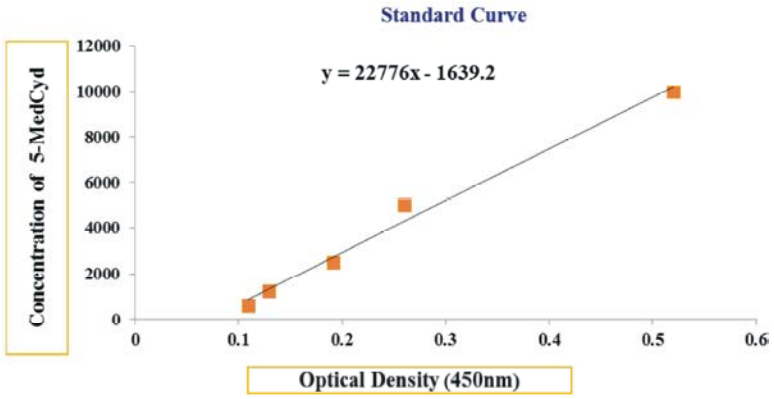

Fig. 1: A standard curve of the relationship between OD and concentration of 5-MedCyd (nM)

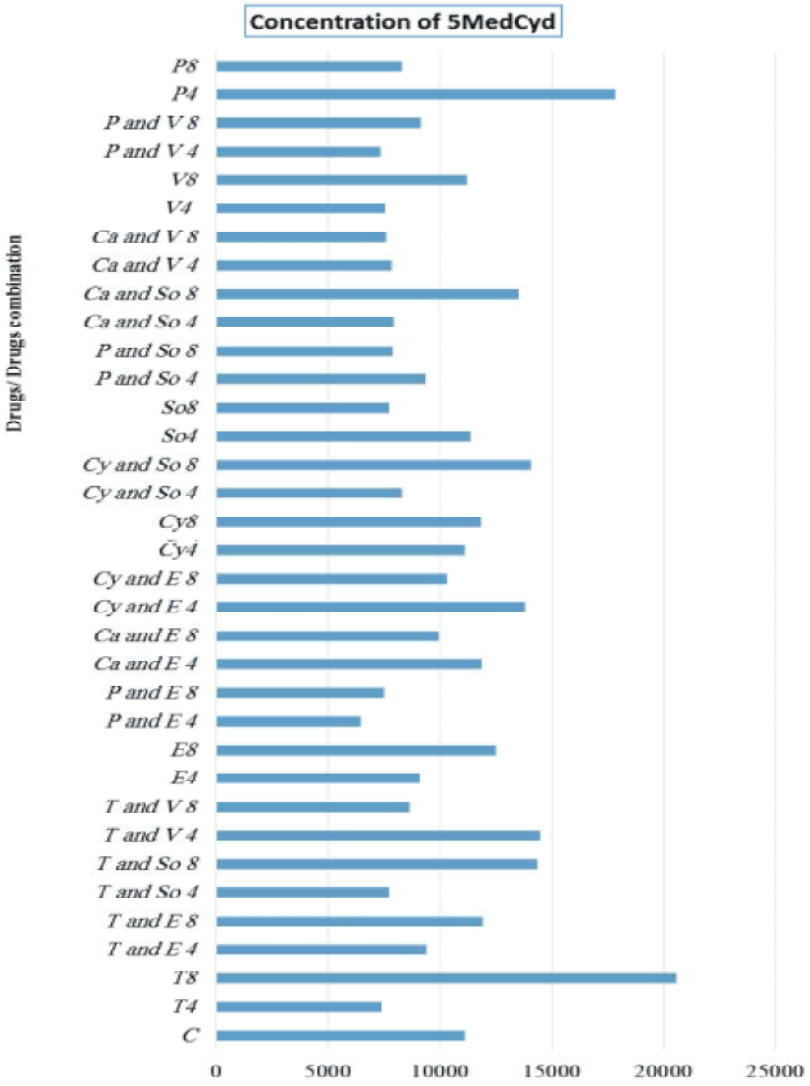

Fig. 2: Levels of 5-MedCyd in nM after treating MCF-7 cells with different drugs. The readings arranged in duration-wise.

C: Control, susp.: Cell suspension, Cy: Cyclophosphamide, So: Sodium Phenylbutyrate, E: Erlotinib, P: Procaine, V: Vorinostat, CA: Carboplatin and T: Temozolomide.

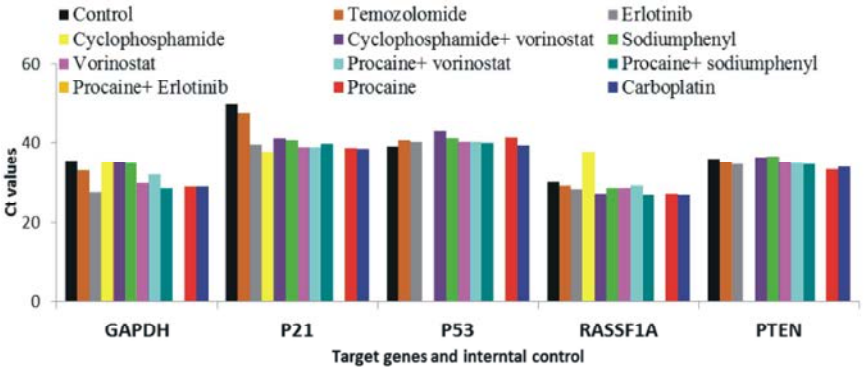

Fig. 3: The cycle threshold values of *P21, P53, RASSF1* and *PTEN* along with *GAPDH* obtained after treating MCF-7 cells with different drugs/drug combinations (set I) for 4 days

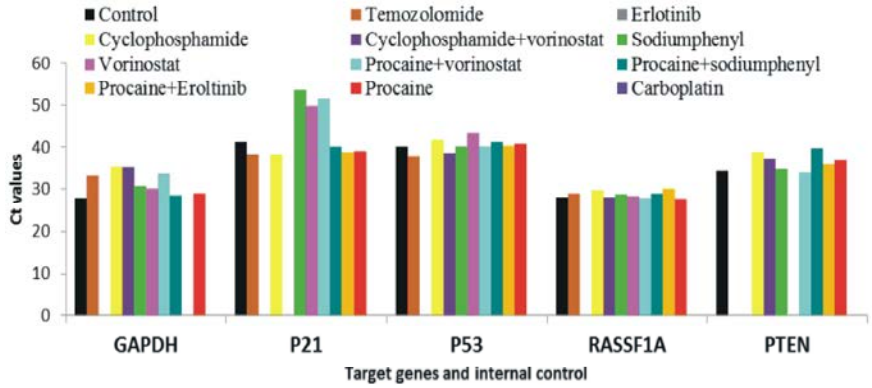

Fig. 4: The cycle threshold values *of P21, P53, RASSF1* and *PTEN* along with *GAPDH* obtained after treating MCF-7 cells with different drug/drug combinations (set I) for 8 days

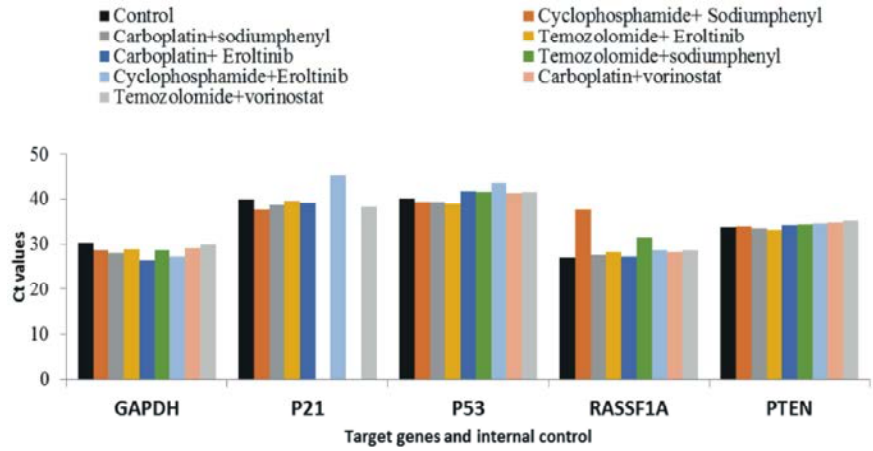

Fig. 5: The cycle threshold values of *P21, P53, RASSF1* and *PTEN* along with *GAPDH* obtained after treating MCF-7 cells with different drug/drug combinations (set II) for 4 days

For the four-day incubation period, data generated indicated that both procaine and sodium phenylbutyrate has resulted in global hypermethylation compared to control [25, 26]. Erlotinib combined with cyclophosphamide or with carboplatin has also resulted in hypermethylation of the genome of MCF-7 breast cancer cells [27-29]. Temozolomide combined with vorinostat has resulted in hypermethylation also in comparison to control [30, 31].

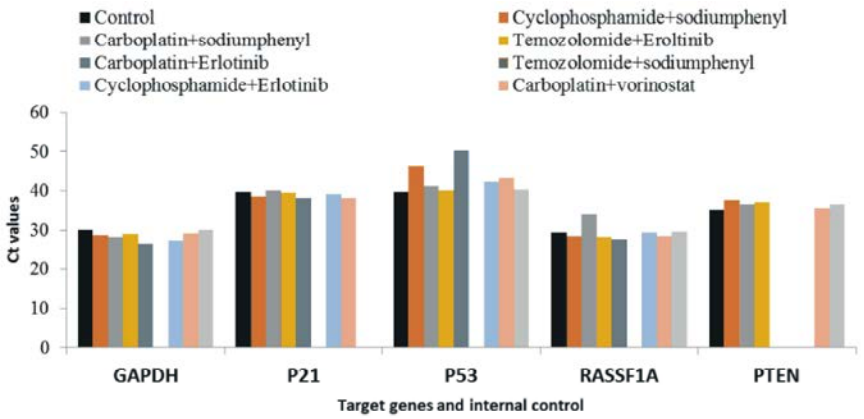

Fig. 6: The cycle threshold values *of P21, P53, RASSF1* and *PTEN* along with *GAPDH* obtained after treating MCF-7 cells with different drug/drug combinations (set II) for 8 days

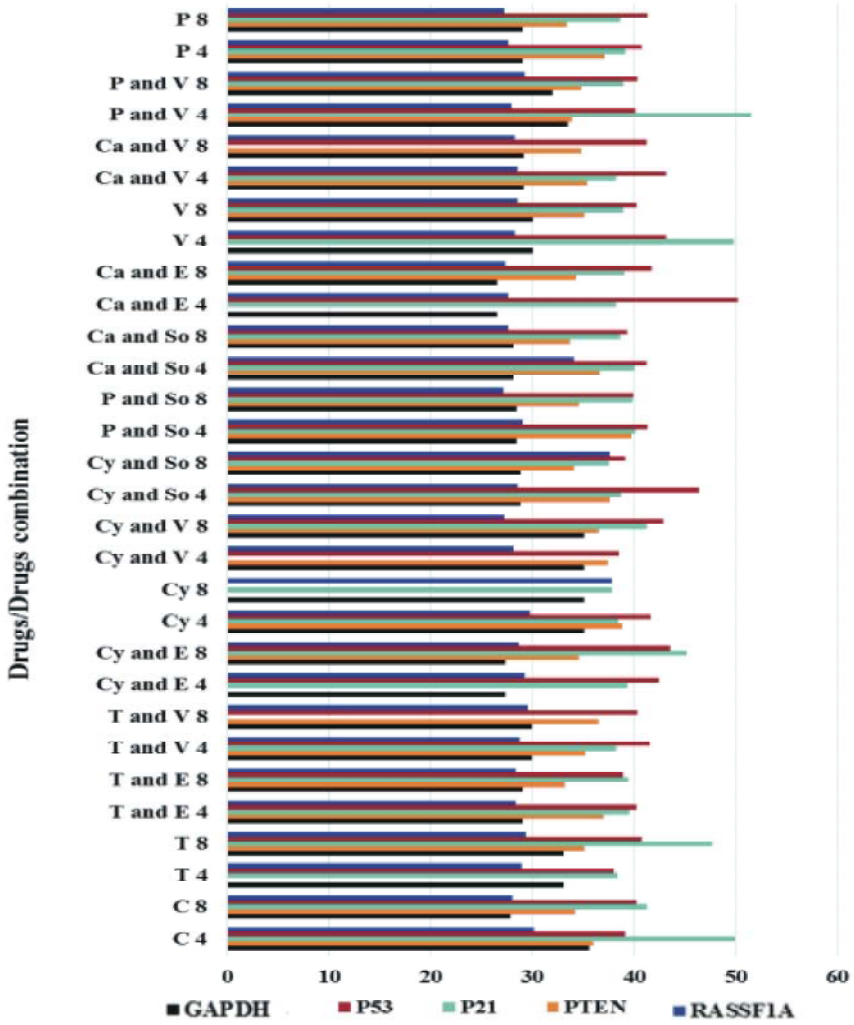

Fig. 7: The overall values of Cts obtained after applying different drugs/drug combinations for the genes GAPDH, P21, P53, PTEN and RASSF1.
C: Control, Cell suspension: susp., Cy: Cyclophosphamide, So: Sodium Phenylbutyrate, E: Erlotinib, P: Procaine, V: Vorinostat, CA: carboplatin and T: Temozolomide.

Real Time PCR: The quantitative Real time PCR (RT-PCR) method was applied to detect the epigenetic-mediated changes in gene expression of *PTEN*, *P53*, *P21* and *RASSF1* in MCF-7 breast cancer cells. Cells were treated with two sets of chemotherapeutic drugs/drug combinations. Both set included vorinostat, procaine, erlotinib, temozolomide, carboplatin, cyclophosphamide, sodium phenylbutyrate, procaine combined with erlotinib, cyclophosphamide combined with vorinostat, procaine combined with vorinostat, procaine combined with sodium phenylbutyrate. Results obtained from the first incubation period (4 days) indicted that *P21*, *P53* and *PTEN* was down regulated, while *RASSF1* was up regulated and this was indicated by the cycle threshold in comparison to the internal control *GAPDH* (Fig. 3). [32-35]. The same profile was obtained with same drugs/drug combination set incubated with cells for 8 days (Fig. 4). These data might indicate the length of time at which drugs being incubated with the cells has no obvious effect. A similar profile has been obtained by several research groups working on different types of malignant cells [20, 36].

The second set of drugs included temozolomide combined with erlotinib, cyclophosphamide combined with erlotinib, cyclophosphamide combined with sodium phenylbutyrate, carboplatin combined with erlotinib, carboplatin combined with vorinostat, carboplatin combined with sodium phenylbutyrate, temozolomide combined with sodium phenylbutyrate and temozolomide with vorinostat. Based on the differences in the cycle threshold, *P21*, *P53* and *PTEN* genes were down regulated while *RASSF1* was up regulated as a result of the treatment. This profile was obtained after incubating the drugs with MCF-7 breast cancer cells for 4 days (Fig. 5). For the longer incubation period (8 days), also the same genes were down regulated; *P21*, *P53* and *PTEN* and on the other hand, *RASSF1* was up regulated (Fig. 6). [31, 37].

CONCLUSION

In the present investigation, we tried to evaluate the role of different chemotherapeutic drugs/drug combination on the epigenetic landscape of breast cancer cells MCF-7. Cell line was treated with two sets of the drugs/drug combinations *i.e*., set I which included vorinostat, procaine, erlotinib, temozolomide, carboplatin, cyclophosphamide, sodium phenylbutyrate, procaine combined with erlotinib, cyclophosphamide combined with vorinostat, procaine combined with vorinostat, procaine combined with sodium phenylbutyrate and set II which included temozolomide combined with erlotinib, cyclophosphamide combined with erlotinib, cyclophosphamide combined with sodium phenylbutyrate, carboplatin combined with erlotinib, carboplatin combined with vorinostat, carboplatin combined with sodium phenylbutyrate, temozolomide combined with sodium phenylbutyrate and temozolomide with vorinostat. The drugs were incubated with the cells for 4 and 8 days. The results obtained indicated the efficacy of the applied drugs in changing the methylation status of the breast cancer cells. Real time PCR data indicated that three tumor suppressor genes *i.e*., *PTEN*, *P21* and *P53* has been down regulated due to the treatments, while *RASSF1* has been up regulated in the same treatments either in the 4-day or in the 8-day incubation periods. Further studies are needed to deeply investigate the mode of action of these drugs on the epigenetic landscape of breast cancer cell line MCF-7.

Conflict of Interests: The authors indicate that there is no conflict of interests.

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