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Changes in Gene Methylation Following Chemotherapy in Breast Cancer Cell Lines

[Meme Kanseri Hücre Soylarında Kemoterapiyi Takiben Oluşan Gen Metilasyon Değişiklikleri]

2. ÖRNEK

Ferda Ari[1],
Rudolf Napieralski[2],
Engin Ulukaya[3]

Uludag University, ,
[1]Science and Art Faculty, Department of Biology, ,
[3]Medical Faculty, Department of Medical Biochemistry, Bursa,Turkey;
[2]Technische Universitaet Muenchen,m rechts der Isar, Department of Obstretrics and Gynecology, Munich, Germany,

Yazışma Adresi
[Correspondence Address]

Doç. Dr. Ferda Ari

Uludag University, Science And Arts Faculty, Biology Department, 16059, Bursa, Turkey
Tel. +90 224 2941822
Fax. +90 224 2941899
E-mail. ferdaoz@uludag.edu.tr

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ABSTRACT

Objective: Epigenetic modulation of gene expression by DNA promoter methylation may contribute to acquired resistance to chemotherapy in cancer cells. Decitabine (5-aza-2'-deoxycytidine), a demethylating agent, may act synergistically with standard chemotherapy regimens to activate epigenetically silenced genes. In the present *in vitro* study, it was investigated the effect of gene methylation level after treatment with decitabine and combination of decitabine with anthracycline-based therapeutics (5-fluorouracil plus epirubicine plus cyclophosphamide; FEC) on breast cancer cells (MCF-7 and MDA-MB-231).

Methods: The effect of decitabine and its combination with FEC on different genes methylation level has been tested in MDA-MB-231 and MCF-7 human breast cancer cell lines. The effect of decitabine on the cell viability was assayed by MTT assay. Methylight real-time PCR and methylation specific PCR were carried out to determine the methylation status of certain genes: DAPK, TMS1, MGMT and the global methylation marker LINE-1.

Results: The LINE-1 methylation status significantly decreased in both cell lines after treatment with the combination of decitabine with FEC. In MDA-MB-231 cells, methylation of the TMS1 and the MGMT gene promoter was significantly reduced by FEC plus decitabine while no effect was observed in MCF-7 cells.

Conclusion: Anthracycline-based therapy regimens in combination with demethylating agents such as decitabine may affect chemotherapy outcome by modulation of apoptosis-relevant genes by methylation. More importantly, this modulation seems to be dependent on the cell type.

Key Words: DNA methylation, breast cancer, apoptosis, decitabine, FEC

Conflict of Interest: The authors do not have any conflict of interest.

ÖZET

Amaç: DNA promotör metilasyonu yoluyla gen ekspresyonunun epigenetik modülasyonu kanser hücrelerinde kemoterapiye karşı direnç neden olabilir. Bir demetile edici ajan olan desitabin (5-aza-2'-deoksisitidin) epigenetikle susturulmuş genleri yeniden aktive ederek standart kemoterapi rejimleri ile sinerjistik etki gösterebilir. Bu in vitro çalışmada, desitabin ve desitabinin antrasiklin-bazlı tedavi (FEC:5-Florourasil+Epirubisin+Siklofosfamid) ile kombinasyonunun meme kanseri hücrelerinde gen metilasyon seviyelerine etkisi araştırıldı.

Metot: Desitabinin tek başına ve FEC ile kombinasyonunun farklı genlerin metilasyon seviyeleri üzerine etkisi insan MDA-MB-231 ve MCF-7 meme kanseri hücre soylarında araştırıldı. Desitabinin hücre canlılığı üzerine etkisi MTT canlılık testi ile çalışıldı. DAPK, TMS1, MGMT ve genel metilasyon göstergesi olan LINE-1 genlerinin metilasyon seviyelerini belirlemek için Methylight realtime PCR ve metilasyon spesifik PCR kullanıldı.

Bulgular: LINE-1 metilasyon seviyesi desitabin ve FEC kombinasyon tedavisinden sonra her iki hücre soyunda da anlamlı olarak azaldı. MDA-MB-231 hücrelerinde, desitabin ve FEC kombinasyonunun TMS1 ve MGMT gen promotöründe metilasyon seviyelerinde azalmaya sebep olduğu gözlenirken aynı etki MCF-7 hücrelerinde gözlenmedi.

Sonuç: Antrasiklin-bazlı kemoterapinin, desitabin gibi bir demetilasyon ajanı ile kombinasyonu metilasyon aracılığıyla apoptozisle ilişkili genlerin modülasyonu neden olarak kemoterapi sonucunu etkileyebilir. Daha da önemlisi, bu modülasyonun hücre tipine bağlı olarak gerçekleşebileceği görülmektedir.

Anahtar Kelimeler: DNA metilasyonu, meme kanseri, apoptozis, desitabin, FEC

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Breast cancer is recognized as the most common malignancy among women. Substantial advances in therapy and diagnosis have enhanced the survival rate of breast cancer patients [1]. Chemotherapy plays a major role in the treatment of patients with cancer, particularly breast cancer. Adjuvant treatment of high risk breast cancer patients with anthracycline containing regimens (5-fluorouracil plus epirubicine plus cyclophosphamide; FEC) has been proven to be highly effective for treating patients with advanced breast cancer [2]. Research programs led to the identification of a variety of therapy option for breast cancer. Epigenetic mechanisms such as DNA methylation are now recognized to play an important role in cancer. Altering the DNA methylation machinery is a potentially powerful approach to cancer therapy [3].

DNA methylation is a covalent modification of the DNA formed by addition of a methyl group at the 5' carbon residue of cytosine in so-called CpG dinucleotide repeats [4]. DNA methylation, once established, acts as a dominant factor in down-regulation of gene expression. Aberrant DNA methylation plays also an important role in carcinogenesis and tumor apoptosis [5]. DNA hypermethylation occurs in many genes in breast carcinogenesis [6]. Gene-specific methylation has also been suggested as a useful tool for prediction of prognosis or response to treatment in early and advanced breast cancer patients [7,8]. Since epigenetic silencing of genes is known to be associated with breast cancer progression [5,9]. Recent studies showed that promoter methylation of genes used as a biomarker for predicting prognosis in breast cancer [10-12].

Several preclinical cell line and animal models have shown a physiological impact of the DNA methyltransferase inhibitor decitabine (5-aza-2'-deoxycytidine, DAC) as a demethylating agent on gene expression and tumor development [13-15]. Decitabine reverses the hypermethylation status of CpG repeats in gene promoters inducing transcriptional reactivation of epigenetically silenced genes, ultimately leading to restoration of apoptosis and inhibition of tumor growth [16-18]. In fact, re-expression of silenced tumor suppressor genes with demethylating drugs will effect inhibition of cancer cell growth in vitro and in vivo [19,20].

In the present report, we employed a human estrogen receptor negative, highly invasive breast cancer cell line (MDA-MB-231) and an estrogen receptor positive, non-invasive breast cancer cell line (MCF-7) to determine the changes in DNA methylation of DAPK (Death-Associated Protein Kinase), TMS1 (Target of Methylation-Induced Silencing 1; apoptosis), MGMT (O6-Methylguanine-DNA Methyltransferase; DNA repair) LINE-1(Long-Interspersed Repetitive Elements; global methylation marker) genes as a response to the combined chemotherapies with decitabine.

Materials and Methods

Chemicals, Anticancer Drugs and Cell Culture

Decitabine was obtained from Sigma (St. Louis, MO). 5-Fluorouracil (5-FU; EBEWE Pharma, Austria), 4-HC (4-hydroperoxycyclophosphamide, the active metabolite of cyclophosphamide; NIOMECH, Germany), and epirubicine (EBEWE Pharma, Austria) were obtained from the Pharmacy of the Uludag University Hospital, representing standard drug regimens normally used for breast cancer treatment. Stock concentrations of each drug were prepared either in PBS (Phosphate Buffer Saline) or in the dilution buffer provided by the drug company. Working dilutions of the drugs were prepared from stock solutions by diluting them in the appropriate culture medium. For each drug, four different concentrations were used and defined as test drug concentrations (TDC). TDC were determined by pharmacokinetic/clinical information and clinical evaluation data [21]. 100% TDC was defined as mean plasma drug concentration assayed after standard FEC dose administration in cancer patients [22]. Hereby, 100% TDC values (in µg/mL) were defined as follows: 5-FU: 22.50, epirubicine: 0.50, 4-HC: 3.0. Drug concentrations used for in vitro experiments were 200, 100, 50, and 25% of TDC.

Breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in RPMI 1640 supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal calf serum (Invitrogen, Paisley, UK) at 37 ˚C in a humidified atmosphere containing 5% CO_2.

MTT Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cell viability assay was performed as previously described [23]. MCF-7 and MDA-MB-231 cells were seeded per well of a 96-well plate in 200 µl culture medium in triplicates at a density of $5x10^3$ cells. After overnight incubation, media were replaced by fresh ones with or without the decitabine. Cells were treated for 24 and 48 h with 1.25-10 µM decitabine. MTT was supplied as a stock solution (5 mg/ml PBS, pH 7.2) and sterile-filtered. At the end of the treatment period, 25 µl of MTT solution was added to each well and then, after another 4 h at 37 ºC, 100 µl of solubilizing buffer (10% SDS dissolved in 0.01 N HCl) was added to each well. After overnight incubation, the absorbance was determined by an ELISA plate reader (FLASH Scan S12, Analytik Jena, Germany) at 570 nm as a read-out for cell viability. Cell viability of treated cells was calculated in reference to the untreated control cells using the formula:

Viability (%) = [100 x (Sample Abs)/(Control Abs)].

DNA Extraction and Bisulfite Modification

Total cellular DNA was extracted by use of the Genomic DNA Puregene Purification Kit (Qiagen, Hilden, Germany); sodium bisulfite conversion of (un)methylated cytosine was performed using the Epitect Bisulfit kit

(Qiagen) according to the manufacturer's instructions, where one µg of DNA was converted by the following PCR thermal cycler conditions: 5 min at 99 °C, 25 min at 60 °C, 5 min at 99 °C, 85 min at 60 °C, 5 min at 99 °C, 175 min at 60 °C and hold at 20 °C. Treated samples were purified and eluted in 80 µl final volume with Tris-buffered elution buffer and stored at -20 °C until further use.

Analysis of Gene Promoter Methylation Status by Methylight Real-time PCR Assays

Bisulfite-converted DNA was analyzed in duplicates by the MethyLight technique as described previously [24] employing the ABI PRISM7700 Sequence Detection System instrument and software (Applied Biosystems, Inc., Foster City, USA). Methylation-specific real-time PCR for the marker genes LINE-1, DAPK, TMS1 and MGMT were performed in a final volume of 20 µL including 10 µl 2x Quantitect Probe mastermix (Qiagen), 2 µL bisulfite-treated DNA, and assay-defined primer and probe concentrations (Table 1). For normalization of input of bisulfite-converted DNA, an Alu1 reference system was used, containing a DNA-methylation status-independent consensus sequence of the most common Alu1 repeat families [25]. The experiment included a no-template control and a positive control with known DNA-methylation status. SssI-treated human chromosomal DNA (Qiagen) was used as a reference of fully methylated cytosine. Primer and probes were purchased from Metabion (Martinsried, Germany), Applied Biosystems (Foster City, USA), or Microsynth (Lustenau, Austria).

Analysis of Gene Promoter Methylation Status by Methylation-specific PCR (MSP) analysis

Aberrant promoter methylation of TMS1 and DAPK gene was determined by the method of methylation specific PCR (MSP), as reported by Herman et al. [29]. MSP distinguishes unmethylated alleles of a given gene on the basis of DNA sequence alterations after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated DNA sequences was then performed.

PCR was performed using CpG WIZ TMS1/ASC and DAP-kinase Amplification Kits (Chemicon International, Canada, USA). Primer set U will anneal to unmethylated DNA that has undergone a chemical modification. Primer set M will anneal to methylated DNA that has undergone a chemical modification. TThe unmethylated or methylated sequence of TMS1 and DAPK are shown in Table 2. PCR conditions of TMS1 and DAPK promoters: 95ºC for 5 min, 40 cycles of 95ºC for 45 s, annealing for 56ºC 45 s and a final extension 72ºC for 60 s. PCR products was mixed with 1.5 µl of loading dye and then run on 2 % agarose gel. Electrophoresis was carried out at 75 V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining.

Statistical Analyses

All statistical analyses were performed using the SPSS 20.0 statistical software for Windows. The TDC were plotted against the corresponding cell viability values using one-way analysis of variance (ANOVA) and the Student's t-test. Mann–Whitney's U-test was used to analyze the association between the methylation statuses of the assessed genes. A value of $p<0.05$ was considered statistically significant. Results are expressed as mean values plus/minus standard deviation.

Results

Effect of Decitabine on cell viability of MCF-7 and MDA-MB 231 cells

The effect of decitabine (1.25-10 µM) was assessed by the MTT viability assay in MCF-7 and MDA-MB-231 breast cancer cells for 24 and 48h. Decitabine resulted in decrease in the cell viability (about 20% percent) at 10 µM after the treatment for 48 h in both cell types (Figure 1).

DNA-Methylation Status of Genes after Anthracycline-Based Therapy and Decitabine

We examined the effects of 100% TDC FEC, 10 µM, decitabine and their combination on DNA methylation status of certain cancer-related genes: DAPK, TMS1, MGMT and LINE-1 in the MDA-MB-231 and MCF-7 breast cancer cell lines by Methylight Realtime PCR Assay.

LINE-1, as a marker for genome-wide methylation status, displays an elevated overall DNA methylation status for untreated MCF-7 cells when compared to MDA-MB-231 cells (60.9 vs 49.7% promoter methylated reference (PMR). The LINE-1 methylation status significantly decreased in both cell lines after treatment with combined FEC/decitabine drug treatment ($p<0.05$) (Figure 2). When FEC or decitabine are used as a single drug regimens, they impacted the overall methylation status of LINE-1 in MDA-MB-231 cells slightly, only except for treatment of MCF-7 cells with decitabine which led to significant reduction of the overall LINE-1 methylation status (Figure 2).

DAPK promoter methylation was different in the MCF-7 and MDA-MB-231 cell lines, displaying a high methylation rate in the MCF-7 cell line but a low one in the MDA-MB-231 cells. In the MCF-7 cell line, the methylation rate of the DAPK promoter was significantly decreased by treatment with FEC, decitabine, or combination thereof. Likewise, in the MDA-MB-231 cells, the low DNA methylation status of the DAPK gene promoter was not significantly affected by the single drugs or combinations thereof (Figure 3).

Different from the above gene promoter DNA methylation status, the TMS1 and MGMT promoters were completely unmethylated in the MCF-7 cells. In MDA-MB-231 cells, methylation of the TMS1 gene promoter

Table 1. Primer and probe sequences (MethyLight systems)

Gene Primer and probe	Sequence 5'-3'	Size of PCR product (bp)	Reference Sequence	Reference
LINE-1				
Forward	GGACGTATTTGGAAAATCGGG	81	X52230.1 1524-1605	[25]
Reverse	AATCTCGCGATACGCCGTT			
Probe	FAM-TCGAATATTGCGTTTTCGGATCGGTTT-BHQ1			
DAPK				
Forward	TCGTCGTCGTTTCGGTTAGTT	67	AL161787.13 47151-47218	[26,27]
Reverse	TCCCTCCGAAACGCTATC			
Probe	FAM-CGA CCA TAA ACG CCA ACG CCG-BHQ1			
TMS1				
Forward	CGT TGG AGA ATT TGA TCGTCG	79	AF184072.1 1287-1366	[28]
Reverse	CCC GTA ACC CTC GCG CAA			
Probe	FAM-AGT TTA AGA AGT TTA AGT TGA AGT TGT TGT CGG TGT CG-BHQ1			
MGMT				
Forward	CGAATATACTAAAACAACCCGCG	122	X 61657 1029-1150	[26]
Reverse	GTA TTT TTT CGG GAG CGA GGC			
Probe	FAM-AATCCTCGCGATACGCACCGTTTACG-BHQ1			
Alu 1				
Forward	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA	98	Consensus sequence	[25]
Reverse	ATTAACTAAACTAATCTTAAACTCCTAACCTCA			
Probe	VIC-CCTACCTTAACCTCCC-MGB			

Note: Primer and probe concentrations were as follows: uPA, 600 nmol/L primer/200 nmol/L probe; PAI-1, 600 nmol/L primer/200 nmol/L probe; LINE-1, 300 nmol/L primer/100 nmol/L probe; DAPK, 600 nmol/L primer/200 nmol/L probe; MGMT, 600 nmol/L primer/200 nmol/L probe; TMS1, 300 nmol/L primer/200 nmol/L probe; and Alu1, 300 nmol/L primer/100 nmol/L probe.
Abbreviations: I: Inosin binding to cytosine or uracile; BHQ: Black Hole Quencher; MGB: Minor groove binder.

Table 2. Primer sequences (MSP systems)

Gene primer and probe	Sequence 5'-3'	Size of PCR product (bp)	Reference
DAPK			
M primer	Forward GGATAGTCGGATCGAGTTAACGTC	98	[30]
	Reverse CCCTCCCAAACGCCGA		
U primer	Forward GGAGGATAGTTGGATTGAGTTAATGTT	106	
	Reverse CAAATCCCTCCCAAACACCAA		
TMS1			
M primer	Forward TTGTAGCGGGGTGAGCGGC	272	[31]
	Reverse AACGTCCATAAACAACAACGCG		
U primer	Forward GGTTGTAGTGGGGTGAGTGGT	207	
	Reverse CAAAACATCCATAAACAACAACACA		

Abbreviations: M: Metylated primer; U: Unmethylated Primer

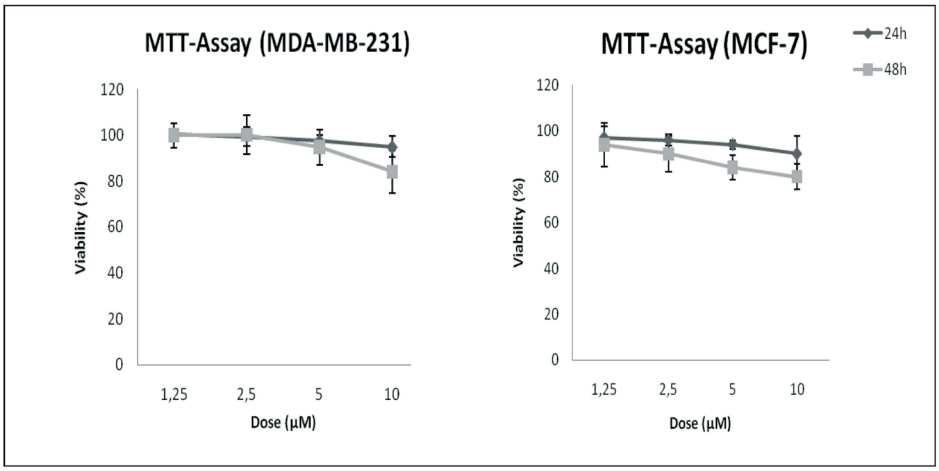


Figure 1. The effects on the cell viability of decitabine for 24 and 48 h. The assays were performed as described in the materials and methods.

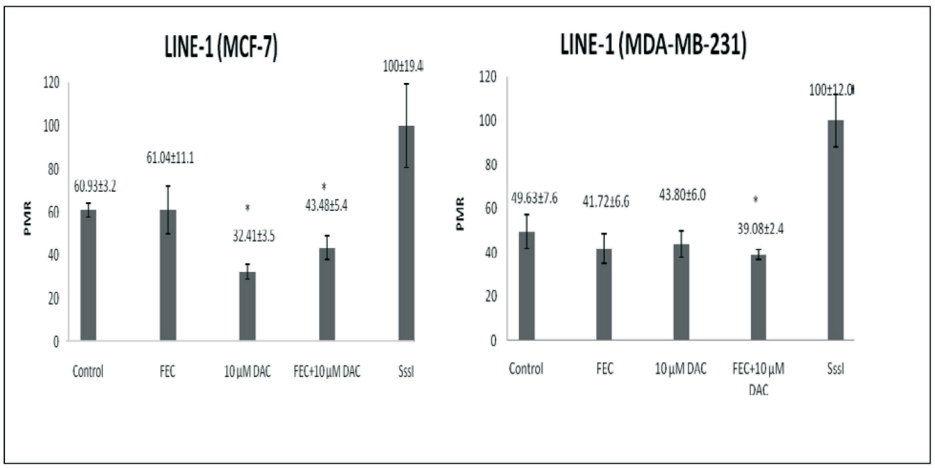


Figure 2. Quantitative assessment of the methylation status of the LINE-1 after treatment with 100% TDC FEC, 10 µM decitabine (DAC), or FEC (100% TDC) plus decitabine (10 µM) for 48 h in MDA-MB-231 and MCF-7 cell lines. SssI, methylated human chromosomal DNA; PMR, percentage methylated reference. *Significant differences of marker levels in relation to untreated control ($p<0.05$) are marked with asterisks.

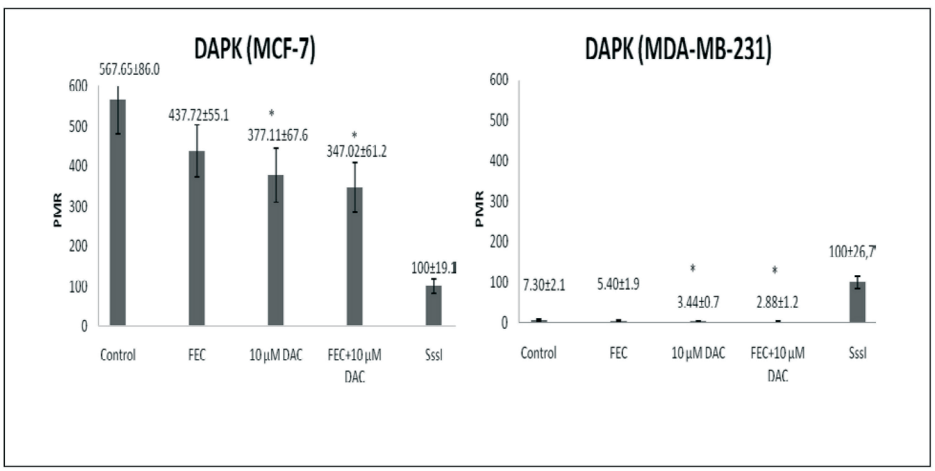


Figure 3. Quantitative assessment of the methylation status of the DAPK after treatment with 100% TDC FEC, 10 µM decitabine (DAC), or FEC (100% TDC) plus decitabine (10 µM) for 48 h in MDA-MB-231 and MCF-7 cell lines. SssI, methylated human chromosomal DNA; PMR, percentage methylated reference. *Significant differences of marker levels in relation to untreated control ($p<0.05$) are marked with asterisks.

was significantly reduced by decitabine, FEC, or combination thereof. Even though that the MGMT promoter showed low methylation levels only in MDA-MB-231 cells, treatment with decitabine alone or in combination with FEC led to further reduction of the methylation signal (Figure 4).

Analysis of TMS1 and DAPK gene promoter methylation by MSP

The analysis of the methylation status of TMS1 and DAPK apoptosis related genes in MCF-7 and MDA-MB-231 breast cancers was carried out by MSP analysis. The results of representative MSP analyses are shown in Figure 5. MCF-7 and MDA-MB-231 cells exhibit partial methylation of analyzed genes. In both cells, the TMS1 gene promoter was demethylated after FEC, DAC and combination (FEC+DAC) treatment. The methylation of DAPK promoter was decrease after treatment of DAC and FEC plus decitabine in both cells.

Discussion

Epigenetic alterations associated with human breast carcinogenesis including hypermethylation of tumor suppressor genes [9] which is also important to predict the risk of developing breast cancer [32,33]. DNA methylation is catalyzed by DNA methyltransferases and inhibition of this mechanism is supposed to be beneficial in the treatment of this malignant disease [34]. Accordingly, DNA methyltransferase inhibitors such as decitabine, which lead to cytosine demethylation, activate epigenetically silenced genes [35-37]. This modification in genes by the effects of decitabine makes it a promising agent for used in cancer chemotherapy. The use of decitabine in combination with other polychemotherapy regimens may lead to even more effective therapy in breast cancer.

In the present study, we investigated methylation status of genes: DAPK, TMS1 (apoptosis-inducers), MGMT (DNA repair), and the global methylation marker LINE-

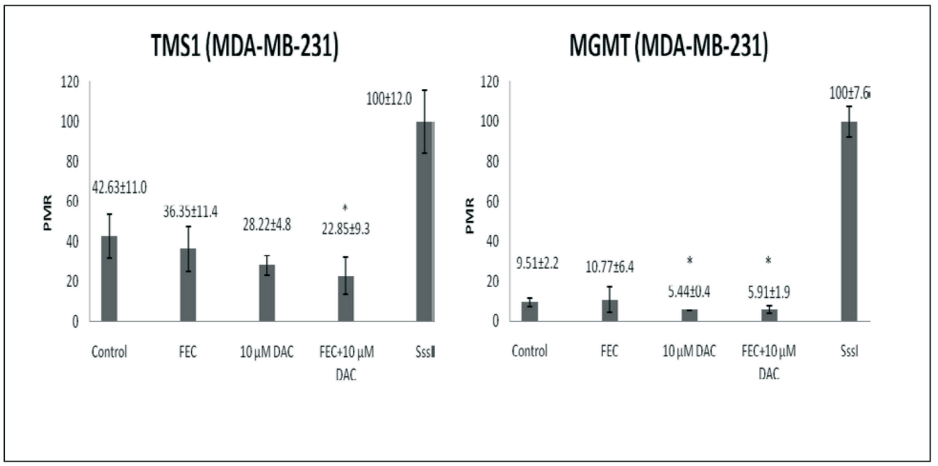


Figure 4. Quantitative assessment of the methylation status of the TMS1 and MGMT after treatment with 100% TDC FEC, 10 µM decitabine (DAC), or FEC (100% TDC) plus decitabine (10 µM) for 48 h in MDA-MB-231 cells. SssI, methylated human chromosomal DNA; PMR, percentage methylated reference. *Significant differences of marker levels in relation to untreated control ($p<0.05$) are marked with asterisks.

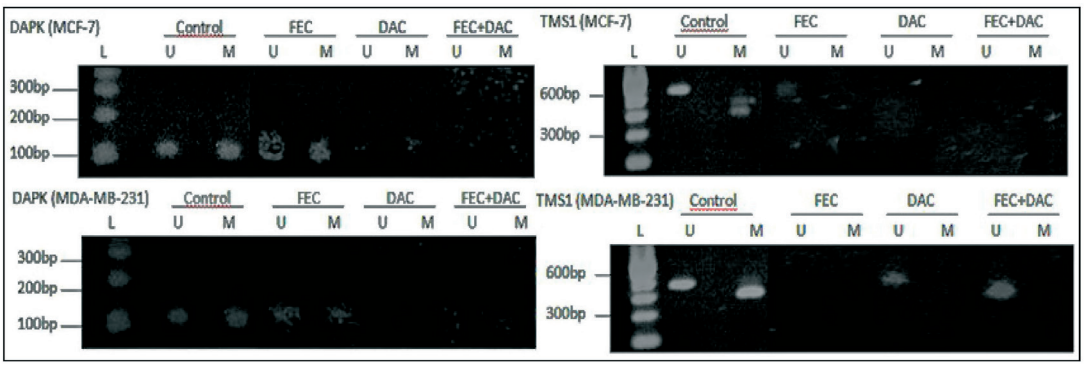


Figure 5. Representative data showing the methylation status of TMS1 and DAPK promoter for MDA-MB-231 and MCF-7 cell lines. M and U are the methylated and unmethylated primers, respectively. U unmethylated DNA, M methylated DNA, indicates molecular weight control (1000pb ladder).

1 using DNA-methylation specific real-time PCR after treatment of decitabine plus FEC chemotherapy regime. In this study, we also examined the effects of decitabine on viability of the breast cancer cell lines MDA-MB-231 and MCF-7. Decitabine lead to decrease of cell viability at 20% percent (10 μM for 48) in both cells. In our previous study, we show that the treatment of MCF-7 and MDA-MB-231 cells with FEC significantly reduced cell viability in a dose-dependent manner. However, the combination of the different doses of FEC with 10 μM decitabine did not impair cell viability levels any further [38].

The methylation level of LINE-1 (long-interspersed) repetitive elements was assessed by MethyLight assay which estimates total genomic DNA methylation [39]. Several clinical studies associated the DNA-methylation status of repetitive sequences like LINE-1 or Sat2 with clinical prognosis in different cancer types [40-42]. We demonstrated that the LINE-1 methylation status is higher in MCF-7 cells compared to MDA-MB-231 and that incubation of the cell lines with decitabine plus FEC resulted in significant reduction of the methylation status.

Activation of general DNA repair pathways are involved in acquired resistance of cancer cells to chemotherapy and the MGMT (O6-methylguanine-DNA methyltransferase) gene, which is involved in the repair of alkylated DNA lesions [43,44]. Promoter methylation of MGMT genes associated with loss of MGMT expression and diminished DNA-repair activity [45]. Recent studies suggested that the MDA-MB-231 cell line exerts MGMT silencing by promoter hypermethylation [46,47]. In our hands, in MDA-MB-231 cells, the MGMT promoter showed only low methylation levels, still, treatment with decitabine alone or in combination with FEC led to further reduction of the methylation signal. In a previously study showed that partial demethylation of MGMT gene prompoter when paclitaxel, adriamycin and 5-fluorouracil combined with decitabine [48].

DAPK and TMS1 are supposed to play a pivotal role in the regulation of apoptosis, inflammatory signaling pathways, and immune response pathways [49-51], which may be implicated in cancer progression [52,53]. In our study, we found high promoter methylation levels of DAPK in the MCF-7 cell line and low methylation ratio in MDA-MB-231 cell line. On the opposite, the TMS1 promoter was hypermethylated in MDA-MB-231, which is consistent with reported studies [54-56]. Correlating results were also reported for DAPK and TMS1 mRNA and protein expression levels in various breast cancer cell lines [54,57]. We found that in cells treated with decitabine, FEC, and the combination regimens, DAPK as well as TMS1 were significantly demethylated in the respective cell lines in a synergistic fashion. In addition, our previously data demonstrate that decitabine plus FEC treatments increased apoptosis than decitabine or FEC treatment alone in MCF-7 cells [38], which may be a result of reactivation of gene expression by promoter demethylation.

Conclusions

In conclusion, these treatments lead to reduction of the methylation status of promoters of the TMS1 and DAPK genes. Furthermore epigenetic effects were also observed for FEC only regimens, which may be related to a kind of epigenetic adaption or selection process in the treated tumor cell population. We concluded that the therapy of FEC plus decitabine, even FEC alone, may affect therapy outcome by upregulation of apoptosis-relevant genes via hypomethylation.

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Conflict of interest: Authors have no conflict of interest.

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