TURKISH JOURNAL of ONCOLOGY

Turk J Oncol 2019;34(2):72–80
doi: 10.5505/tjo.2019.1866

ORIGINAL ARTICLE

Investigating the Effects of DNA Demethylation in Chemotherapy Resistance by ChIP-on-Chip Method

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OBJECTIVE

This study aimed to determine the epigenetic basis of drug resistance mechanisms developed in MCF-7 breast cancer cell line that is resistant to an anticancer agent paclitaxel. Thus, we investigated the effects of the changes in DNA level on gene expression profile and proposed methods of inhibiting resistance by DNA modifications.

METHODS

We investigated the epigenetic basis of acquired drug resistance in whole genome by comparing chromosome immunoprecipitation in paclitaxel-resistant MCF-7 (MCF-7/Pac) cells and in drug-sensitive (MCF-7/S) cells. For this analysis, DNA samples from both cell lines were immunoprecipitated and labeled with Cy3 and Cy5 fluorescent dyes. Hybridization and array scanning was performed with Agilent all-Genome Microarray platform that was designed to detect DNA methylation. The obtained high-throughput information was analyzed with a bioinformatics analysis program.

RESULTS

The results showed that demethylation and epigenetic modulation of the DNA regions encoding 90 genes are significant in the development of multiple drug resistance (MDR) in breast cancer. Some of these genes, ICAM4, COX6B2, ITGB8, SLC39A4, TUBB2C, COL6A1, DAPK1, RUNX3, SLC35F3, and MAP6, are important players in the development of drug resistance and cancer stem cells.

CONCLUSION

Studies on reversing multidrug resistance can be carried out by DNA modification or methylation of target genes regions on DNA. The results presented in this study may shed light on drug development studies to make DNA modifications.

Keywords: Demethylation; epigenetics; multidrug resistance; MDR; MCF-7; paclitaxel.

Introduction

Stable and inherited changes in gene expression without any change in DNA sequences are described as epigenetic alterations. Generally, modulation occurs on DNA or in chromatin by covalent binding and modifications. Epigenetics causes the expression of genes in genetically identical cells and organisms in different forms and show phenotypic differences.[1]

The most emphasized mechanisms is the DNA methylation, which is the hereditary change caused by the addition of methyl group to the 5′ end of the cytosine followed by the guanine nucleotide by the catalysis of enzyme DNA methyl transferase.[2] DNA

Received: March 03, 2019
Accepted: March 04, 2019
Online: May 28, 2019

Accessible online at:
www.onkder.org

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demethylation occurs by non-methylating the DNA strand after replication or during development by a replication-independent process. The impaired signaling in cancer cells may result in stable silencing of downstream targets regulated by epigenetic mechanisms.[3] Methods such as methylation-specific PCR, HELP (HpaII tiny fragment Enrichment by Ligation-mediated PCR) assay can detect DNA methylation. Another approach in the determination of methylation level named "ChIP-on-chip" was developed by ChIP (chromatin immunoprecipitation) method.[4] In this method, the double-stranded DNA is separated and treated with methyl cytosine antibodies. Once the DNA has been labeled, it is hybridized with the microarray platform affixed with specific probes covering the whole human genome.

Drug resistance, which is acquired or intrinsic in the patient post-treatment or pre-treatment period, severely prevents success in cancer chemotherapy. This case is called multidrug resistance (MDR).[5] Resistance against chemotherapy prevents many anticancer drugs to show the expected effects on the patients and causes progression of the disease. Increased drug doses lead to increased side effects and limited treatment.

This study aimed to determine the effects of DNA demethylation and epigenetic bases of drug resistance mechanisms developed in MCF-7 breast cancer cell line that is resistant to anticancer agent paclitaxel (MCF-7/Pac). The resistant cell line was developed by treatment of MCF-7 breast cancer cells with paclitaxel by dose increments. Paclitaxel is an anticancer drug that inhibits cell division by interfering microtubules during mitosis. Changes in gene expression profiles of paclitaxel-resistant MCF-7 (MCF-7/Pac) cells compared to drug-sensitive (MCF-7/S) cells have been presented in previous publications.[6,7] Recently, it was reported that MCF-7/Pac cells exhibit the major features of breast cancer stem cells.[8] Therefore, determination of DNA methylation levels of MCF-7/Pac cells with respect to MCF-7/S cells will reveal the effects of DNA methylation in breast cancer stem cells. In this study, epigenetic bases of developing resistance were proposed with preliminary findings.

Materials and Methods

Cell Culture Conditions

MCF-7/S cell line sensitive to anticancer drugs and paclitaxel-resistant MCF-7/Pac cell line was used in the study.[9] RPMI 1640 containing 10% (v/v) serum (fetal bovine serum, FBS) and 2 mM L-glutamine was used as the culture medium of the cells. To prevent microbial infection, gentamycin (1 mg/ml) was added to the medium, and the cells were incubated at 37°C, with 5% CO_2 incubator. Adhesive cells were transferred into new culture medium with trypsin-EDTA when they covered 70% of the cell culture vessel. Paclitaxel-resistant cells were established by adding stepwise increasing paclitaxel in to the culture medium in two years. This cell line was used as rational model for drug resistance in breast cancer.[9]

DNA Isolation and Immunoprecipitation

The experiments were conducted according to Agilent G4170-90012_Methylation_Protocol_v.2.0 in the laboratories of Selçuk University ILTEK. The genomic DNA isolation from MCF-7/S and MCF-7/Pac cells (1×10^6 cells from each) was performed using the genomic DNA isolation kit (QIAamp DNA mini kit, QIAGEN). The quality control procedure was performed by spectrophotometric measurements (OD260nm/OD280nm, Nanodrop-Thermo) and agarose gel electrophoresis. Genomic DNA (5 µg from each) was fragmented by ultrasonication by ultrasonicator (using micro probe, Heidolph) that was set output power to 70%. The DNA fragmentation was ensured by sonicating the DNA samples for 5 s, five cycles on ice by holding 5 s between each cycle intervals (to prevent heating and foaming). For immunoprecipitation procedure, Dynabeads Pan Mouse IgG magnetic beads were precipitated by magnetic stand (DynaMag-2 Magnet, Life Technologies), and the beads were bound to ChIP grade anti-5-Methyl Cytidine antibody (Abcam) by incubating in a rotator (VWR) at 4°C incubator. The fragmented DNA samples were then hybridized with the magnetic bead bound 5-methylcytidine antibody in immunoprecipitation buffer (Triton X, yeast t-RNA, PBS) to let the binding of methylated DNA fragments on to the magnetic antibodies (in rotator, overnight at 4°C incubator). Finally, the methylated DNA fragments were sorted on magnetic stand. The methylated DNA samples and reference DNA samples (that were not immunoprecipitated) were eluted by elution in TE and SDS solution. DNA fragments were extracted through phenol-chloroform extraction method by use of MaXtract High Density tubes (QIAGEN). The aqueous phase was collected, and DNA was precipitated by adding the precipitation solution that constitutes NaCl (200 mM), glycogen 20 µg/mL, and pure ethanol. DNA was precipitated by incubating the mixture at 4°C and centrifugation at 12.000 g for 3 min (Hettich). Finally, the DNA pellets were air dried. The quality of eluted methylated DNA

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and reference DNA samples of MCF-7/Pac and MCF-7/S were measured by Nanodrop (Thermo).

Fluorescent Labeling of DNA Samples

Methylated and reference DNA fragments were labeled using the SureTag DNA Labeling Kit (Agilent). Briefly, DNA samples were mixed with random primers and incubated at 95°C for 3 min on heating-cooling dry block (Biosan). While DNA samples were denatured, they were labeled by Exo Klenow enzyme, dNTPs. and fluorescently labeled dUTPs (Cy-3 and Cy-5 labeled) at 37°C for 2 h in PCR machine (Biorad). After the reaction was stopped at 65°C, labeled DNA fragments were purified by purification columns, and fluorescence binding success was determined by spectrophotometric measurements (Nanodrop, Thermo). Specific activity and labeled DNA amounts were calculated using the following formulas:

Specific activity=pmol dye per µL of dye/µg per µL DNA
Yield (µg)=DNA concentration (ng/µL)×sample volume (µL)/1000 ng/µg

Hybridization of Labeled DNA on Arrays

Since DNA labeling quality was good (yield >2.5 µg and appropriate specific activity), the labeled DNA fragments were hybridized on the array platforms (Human DNA Methylation Microarray, 1×244K (HD)). Briefly, the hybridization master mix was prepared for four samples (duplicates for each cell line). Hybridization master mix constituted Human Cot-1 DNA for internal control, CGH (chromosomal genomic hybridization) blocking agent, HI-RPM hybridization buffer, and deionized formamide. Hybridization master mix (420 µL for 1-pack microarray format) was combined with Cy3- and Cy5-labeled DNA samples (40 µL each) in one tube for each cell line. Two replicates of hybridization mixtures were prepared for MCF-7/S and MCF-7/Pac cell lines and incubated at 95°C for 3 min and 37°C for 30 min in thermal cycler (Biorad). The hybridization solution should be kept at 37°C until it is loaded on to the array slides. Hybridization solution was slowly dispensed on to the gasket slide carefully preventing the overflow of the solution out of the gasket chamber. Then the printed microarray slide (Agilent, Unrestricted Amadid Chip-on-Chip 1×244K) was put on to the gasket slide (barcode number should be outside). Finally, the microarray slide was assembled in the slide chamber. The hybridization oven was set to 67°C, and the chambers were placed in to the array holders in a balanced way. The hybridization proceeded for 40 h at 20 rpm rotation speed. Two repeats were performed for each group (MCF-7/S and MCF-7/Pac), (n=2).

Washing and Scanning

The washing equipments were washed with acetonitrile and ultrapure water previously. ChIP-on-chip wash buffer 2 was prewarmed in a coplin jar before disassembling the slide chambers (37°C). The slides were disassembled in ChIP-on-chip wash buffer 1, in to a separate coplin jar. Then the slides were put into another jar that contains wash buffer 1 for 5 min at room temperature (a magnetic bead was put at the basement of the jar to provide continuous stirring by magnetic stirrer). Arrays were immediately air dried, and ozone covers were put on to prevent adverse effects of the ozone on to the slides. During the whole procedure, slides should be kept by forceps. Sure Scan Microarray Scanner (Agilent), which was in the laboratory of Selçuk University ILTEK, was turned on to warm up the lasers. The G4900DA SureScan Microarray Scanner System Microarray Scan Control Software 9.1 (Agilent) was run, and Protocol Agilent HD-CGH was selected. The slides were immediately inserted into the scanner as the scanner was ready to scan the arrays. The machine recognized the barcode of the array. The green and red lasers excited the fluorescent dyes, and then photomultiplier tube detector detected the data. After scan protocol was completed, the Feature Extraction software v11.0.1.1 was operated for quality control (QC). The QC reports were generated using the grid Human DNA Methylation Microarray 244k-023795-D-F-20111018.

Advanced Analysis and Statistics

The scan data of each cells were analyzed by Genomic Workbench ver6.0 (Agilent) program. Z-score algorithm was used for calculations. Gaussians were fitted to the bimodal log ratio distribution. Significant changes were determined between the groups (MCF-7/S and MCF-7/Pac) by Student's t-test ($p<0.05$). Finally, the significant results were listed, and the demethylation levels were calculated by calculating the fold change values and presented as the logarithm of the fold change ratios (MCF-7/Pac/MCF-7).

Results

Proliferation of the Cells and Preparation of DNA Fragments

The cell lines were cultured, and DNA isolation and QC of DNA samples were performed. Figure 1 ex-

hibits the image of the MCF-7/Pac cells under an inverted phase contrast microscope (Leica). In Figure 2, agarose gel electrophoresis images of the genomic DNA samples and fragmented DNA smears are presented. The purity, concentration, and quality of the DNA samples isolated from the cell lines were found to be suitable for the continuation of the microarray protocol (Table 1). Table 2 presents the results of the specific activity and yield calculations of fluorescently labeled methylated and reference DNA fragments. These results showed that it was possible to continue experiments with the labeled DNA fragments of suitable concentrations. According to these results, microarray hybridization and scanning were performed.

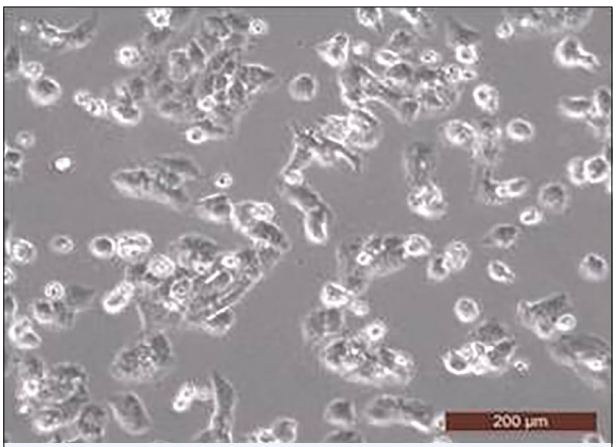

Fig. 1. Invert microscope image of MCF-7/Pac cells.

Table 1 Quality and yield of genomic DNA samples from cell lines

	Concentration ng/µL	Yield µg	Quality 260/280	260/230
MCF7/S1	143.60	7.90	1.82	2.23
MCF7/S2	141.50	7.78	1.88	2.17
MCF7/Pac1	158.10	8.69	1.81	2.04
MCF7/Pac2	177.40	9.76	1.70	1.82

Hybridization, Washing, and Scanning Protocols

The hybridization, washing, and scanning protocols were completed; and the QC reports were created for each arrays with the Feature Extraction program (Agilent). The results obtained for all the scanning protocols were reported as "good." The scan image and grid results obtained after scanning are presented in Figure 3. The quality analysis of the repetitive arrays for each group yielded favorable results for further analysis (Figs. 4, 5).

Advanced Analysis by Genomic Workbench Program

The raw data obtained after scanning the arrays were analyzed by Genomic Workbench ver6.0 (Agilent). The fluorescence values were analyzed, and array results for MCF-7/S and MCF-7/Pac were compared. As a result, when the drug resistance was developed, decreases in the methylation level of the gene regions on DNA (demethylation) were identified. The fold change values were calculated to reveal the demethylation levels after acquired drug resistance. The logarithms of

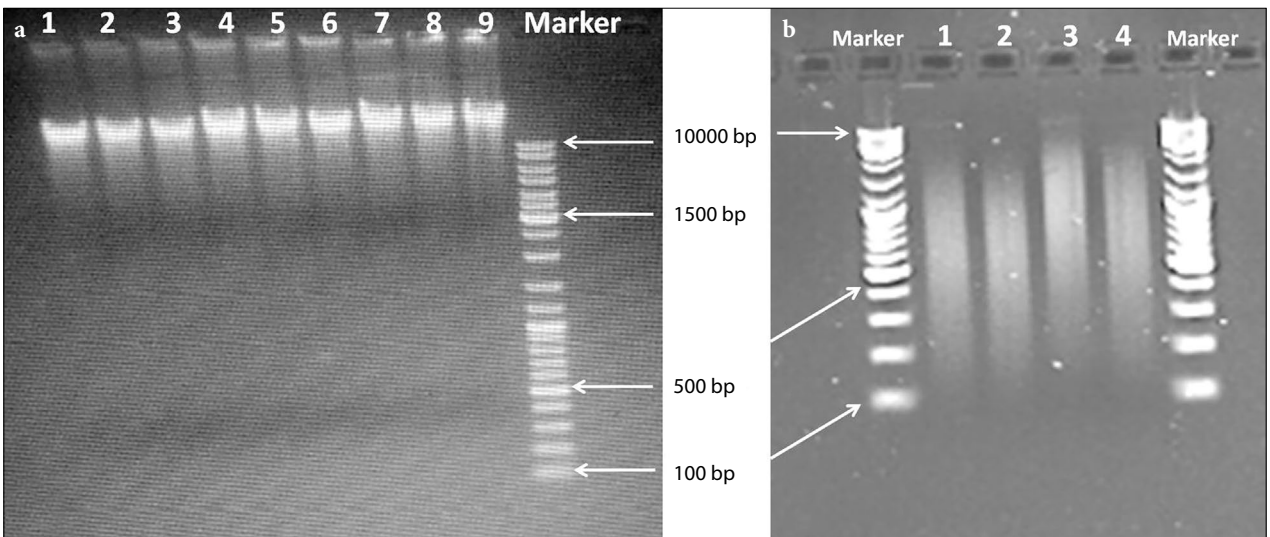

Fig. 2. (a) Agarose gel electrophoresis image of genomic DNA from cells; 1–5: MCF-7/S, 6–9: MCF-7/Pac, Marker: High range DNA ladder: 100 bp–10 kb. (b) Fragmented DNA smear from DNA samples; 1,2: MCF-7/S, 3–4: MCF-7/Pac, Marker: High range DNA ladder: 100 bp–10 kb.

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doi: 10.5505/tjo.2019.1866

Table 2 Cy5- and Cy3-labeled DNA concentrations and yields

Cy 5 labeled ChIP DNA			Cy 3 labeled reference DNA		
Specific activity (pmol/µg)		**Yield (µg)**	**Specific activity (pmol/µg)**		**Yield (µg)**
MCF-7/S-1	9.80	27.1	Ref. MCF-7/S-1	11.0	41.1
MCF-7/S-2	9.60	28.3	Ref. MCF-7/S-2	12.0	43.9
MCF-7/Pac-1	7.50	22.5	Ref. MCF-7/Pac-1	10.5	38.4
MCF-7/Pac-2	9.00	21.4	Ref. MCF-7/Pac-2	9.50	35.4

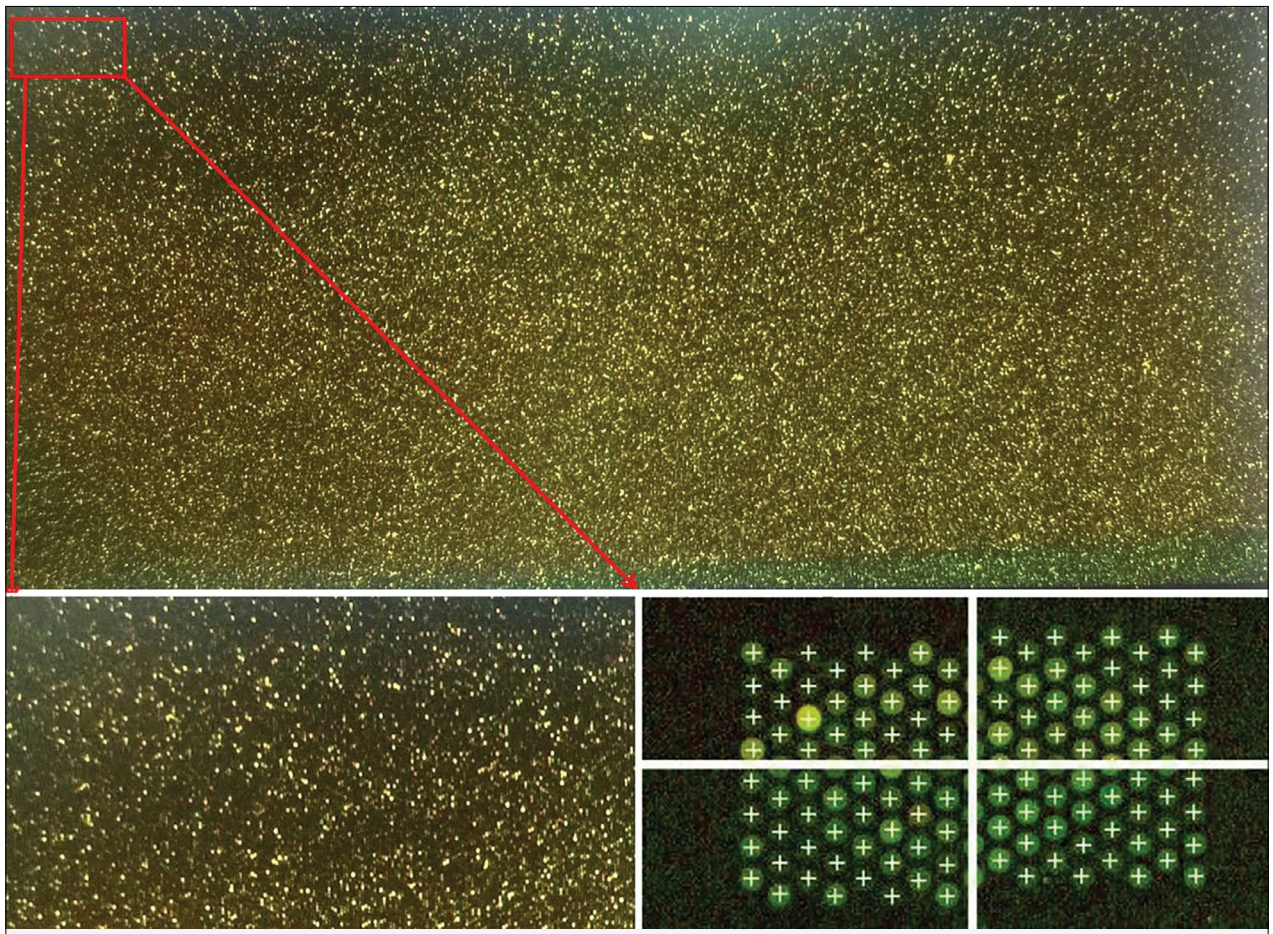

Fig. 3. Representative microarray scan photo and quality check of array corners (MCF-7/Pac).

the results were calculated and significant results were listed ($p<0.05$) (Table 3). At the end of the analysis, we identified significant demethylation in 90 gene regions in paclitaxel-resistant breast cancer cells with respect to drug-sensitive breast cancer cell line. The demethylation levels were in range of 10.35–2.14 values. The demethylated ICAM4, COX6B2, ITGB8, SLC39A4, TUBB2C, COL6A1, DAPK1, RUNX3, SLC35F3, and MAP6 gene regions are related to cancer metastasis and cancer stem cell development.

Discussion

Cancer is a clinical problem, and it seriously affects human health and life. Although important studies have been carried out in the development of new chemotherapeutic agents, cancer still affects millions of patients worldwide. Chemotherapy-resistant breast cancer stem cells are known to make the treatment of the disease difficult.[8,10] Recent studies focus on epigenetic events and MDR1 transcription changes [11,12,13] due to epi-

Table 3 Significant demethylation levels of the gene regions in MCF-7/Pac cells (MCF/Pac vs. MCF-7/S ratio of signal logarithms, p<0.05, genes were annotated according to probe numbers.)

Probe name	Gene	Demethylation level	Probe name	Gene	Demethylation level
A_17_P17107441	ICAM4	10.35	A_17_P16147607	EPPK1	2.97
A_17_P16897418	IRX3	9.36	A_17_P02636320	CHST2	2.91
A_17_P11002493	COX6B2	8.71	A_17_P07432271	TLX1	2.89
A_17_P10977774	PNMAL1	6.74	A_17_P16278514	PTGDS	2.88
A_17_P17028063	FASN	6.50	A_17_P16760152	SNRPN	2.87
A_17_P11852323	HNRNPH2	5.77	A_17_P24840452	HLA-DRB5	2.86
A_17_P15009776	MMP23A	5.75	A_17_P10865025	UHRF1	2.86
A_17_P27241394	LPAR1	5.59	A_17_P01898996	WNT6	2.85
A_17_P15599963	PAPSS1	5.27	A_17_P01281692	LBX2	2.79
A_17_P16269780	SETX	5.21	A_17_P23515292	NEUROG2	2.78
A_17_P15046194	TXLNA	5.13	A_17_P31459414	GPX4	2.71
A_17_P09170423	FAM158A	5.12	A_17_P26552644	REXO1L2P	2.69
A_17_P04865469	TBX18	5.02	A_17_P01844528	FZD5	2.69
A_17_P27336397	NTNG2	4.95	A_17_P16047954	PTPRN2	2.67
A_17_P10810700	NETO1	4.52	A_17_P16468290	CCND1	2.64
A_17_P15033838	KLHDC7A	4.49	A_17_P16631901	NUFIP1	2.58
A_17_P01379445	POU3F3	4.45	A_17_P16607147	ULK1	2.56
A_17_P05346160	ITGB8	4.44	A_17_P15358368	HOXD9	2.55
A_17_P09888740	SYNGR3	4.28	A_17_P30001382	TMEM121	2.52
A_17_P16149974	SLC39A4	4.21	A_17_P17093035	ARID3A	2.52
A_17_P10485826	CBX4	3.81	A_17_P16242726	HIATL1	2.51
A_17_P17152705	NOSIP	3.76	A_17_P17094542	DAZAP1	2.50
A_17_P16982751	SC65	3.73	A_17_P01110105	CDC42EP3	2.50
A_17_P16047426	PTPRN2	3.67	A_17_P31464357	MOBKL2A	2.46
A_17_P03114655	HOPX	3.60	A_17_P11375307	SIM2	2.45
A_17_P10175143	ZCCHC14	3.57	A_17_P20003247	SAMD11	2.42
A_17_P17276338	PISD	3.45	A_17_P09986653	COX6A2	2.37
A_17_P17194832	SYS1-DBNDD2	3.45	A_17_P15011388	SKI	2.35
A_17_P15587902	CDS1	3.44	A_17_P05171451	AKAP12	2.31
A_17_P31623096	PPFIA3	3.32	A_17_P10345122	TBKBP1	2.29
A_17_P07845095	DRAP1	3.31	A_17_P16933459	NXN	2.29
A_17_P17157815	ZNF816A	3.25	A_17_P00110295	TMEM200B	2.28
A_17_P04646304	PRRT1	3.20	A_17_P09533565	AHNAK2	2.28
A_17_P20005955	MXRA8	3.20	A_17_P00096239	RUNX3	2.28
A_17_P31345484	ZNF532	3.19	A_17_P00870602	SLC35F3	2.26
A_17_P12033051	ZNF275	3.19	A_17_P27342502	ADAMTS13	2.25
A_17_P17199320	KCNG1	3.13	A_17_P11430491	GP1BB	2.24
A_17_P17234564	SIM2	3.13	A_17_P16283000	DIP2C	2.23
A_17_P16279555	TUBB2C	3.13	A_17_P17191219	PPP1R16B	2.23
A_17_P17412386	GABRE	3.11	A_17_P07881315	MAP6	2.23
A_17_P16615763	ZDHHC20	3.11	A_17_P27143285	NXNL2	2.22
A_17_P23032802	GAK	3.11	A_17_P16364294	DYDC2	2.20
A_17_P32082386	COL6A1	3.02	A_17_P17235496	KCNJ6	2.19
A_17_P09889457	CASKIN1	3.00	A_17_P16463611	CD248	2.15
A_17_P16236141	DAPK1	3.00	A_17_P22953020	SOX2	2.14

genetic alterations. It is known that methylation causes decrease in gene expression level, and demethylation may be a way to turn the gene on. Baker and El-Osta reported that epigenetic changes of ZFP (zinc finger protein-encoding gene regions) may be important in the development of drug resistance.[11] Our findings also show that different ZFP (zinc finger protein) proteins are demethylated in the development of resistance

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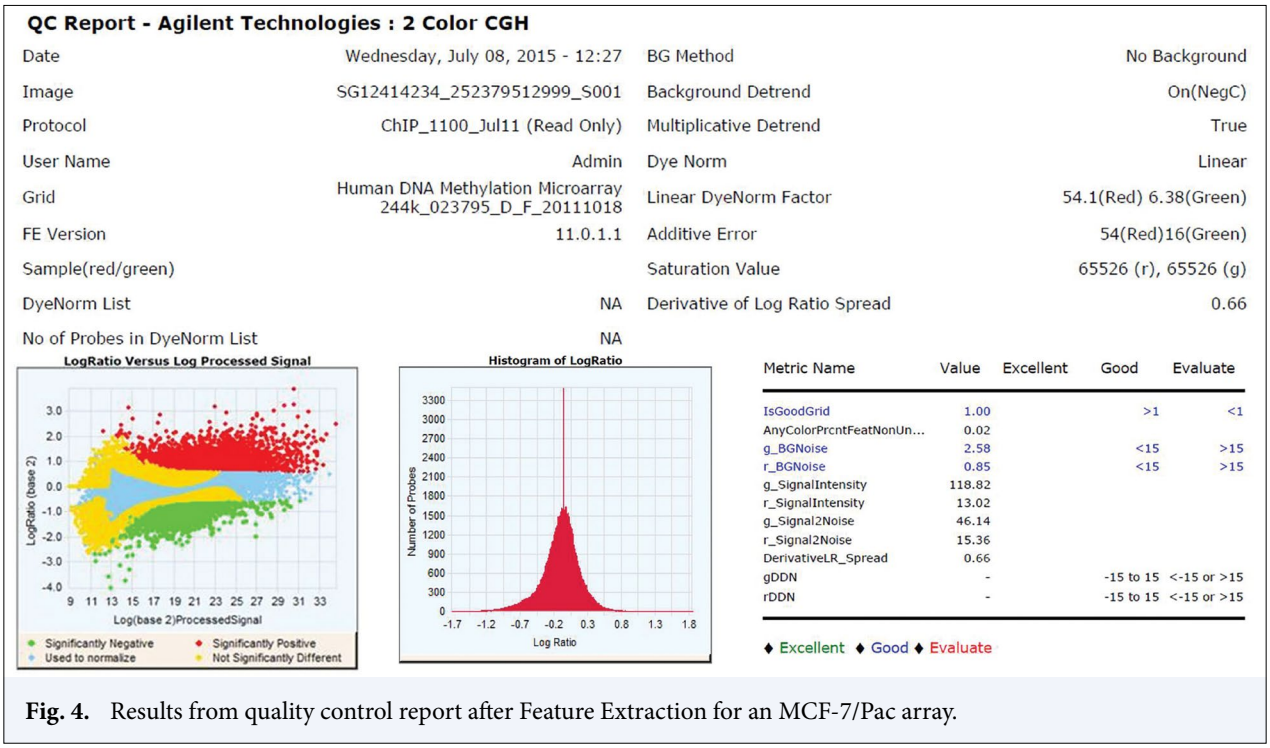

Fig. 4. Results from quality control report after Feature Extraction for an MCF-7/Pac array.

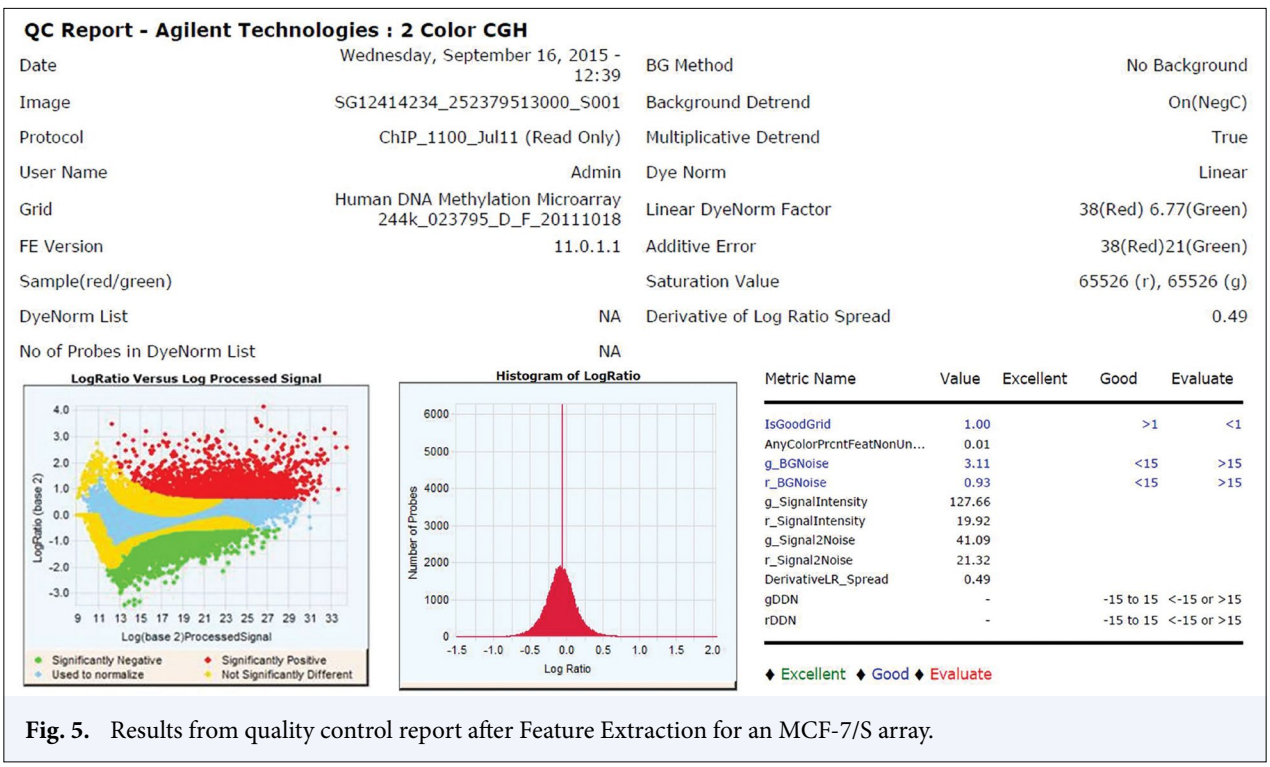

Fig. 5. Results from quality control report after Feature Extraction for an MCF-7/S array.

(Table 3). Demethylated ICAM4, COX6B2, ITGB8, SLC39A4, TUBB2C, COL6A1, DAPK1, RUNX3, SLC35F3, and MAP6 gene regions may attract attention during selection of target genes.

It was previously claimed that intercellular adhesion protein-coding gene ICAM4 may be a breast cancer susceptibility gene, and genetic variants in the DNA loci was correlated with disease severity and metasta-

sis.[14] COX6B2 encodes the subunitVIb polypeptide 2 of cytochrome-C-oxidase enzyme that functions in respiratory chain. Ayyasamy et al. demonstrated that COX6B2 was downregulated in breast cancer.[15] Here we found that COX6B2 was demethylated about 8.71 folds. Therefore, this result needs further investigation. Integrin beta-8 is an important player of drug resistance [16] in parallel, microarray results revealed that ITG8B coding DNA was demethylated 4.4 folds in paclitaxel-resistant breast cancer cells. Solute carrier protein (SLC39A4) was proved to be a biomarker and an important protein in tumor development.[17] Here we found that SLC39A4 was demethylated about four folds in drug-resistant breast cancer cell line. Here, we can propose that SLC39A4 may be a target protein in MCF-7/Pac cell line that expresses the features of breast cancer stem cells. Tubulin beta family members are the targets of paclitaxel that inhibits mitotic division. We previously reported that expression of TUBB genes is upregulated in MCF-7/Pac cells.[18] We found here that TUBB2C gene coding DNA was demethylated about three folds. We also previously found that DAPK1 and COL6A1 genes were over-expressed in MCF-7/Pac cell line.[19] In this study, we confirmed that upregulation of those genes may be due to the demethylation process. In this study, the findings of our previous cDNA microarray analysis are confirmed by ChIP-on-chip microarray method.[20]

Conclusion

The listed 90 genes have merit to be further investigated to eliminate the uncertainty about some of them. New research questions may be asked for further research: can the clinical course of chemotherapy affect demethylation? What is the effect of an active DNA demethylase enzyme associated with the MDR1 promoter on the development of drug resistance? Answers to these questions and the results derived from this paper will allow identifying the association between demethylation and alterations in gene expression levels in drug resistance in breast cancer and for developing new methylating or demethylating therapeutic agents.

Acknowledgment: We acknowledge support from Selçuk University BAP.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors have no conflict of interest pertaining to this manuscript.

Ethics Committee Approval: Since cell lines were used in this study, ethics committee permission is not required.

Financial Support: Selçuk University BAP project (number 14401033).

Authorship contributions: Concept – M.D.K.; Design – M.D.K.; Supervision – M.D.K.; Materials – G.K.; Data collection &/or processing – G.K.; Analysis and/or interpretation – G.K.; Literature search – M.D.K.; Writing – M.D.K.; Critical review – M.D.K.

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