Mol. Cells *33*, 127-133, February 29, 2012
DOI/10.1007/s10059-012-2182-8

Molecules and Cells

Expression Profiling after Induction of Demethylation in MCF-7 Breast Cancer Cells Identifies Involvement of TNF-α Mediated Cancer Pathways

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Epigenetic methylation change is a major process that occurs during cancer development. Even though many tumor-related genes have been identified based on their relationship between methylation and expression, few studies have been conducted to investigate the relevant biological pathways involved in these changes. To identify essential pathways likely to be affected by methylation in breast cancer, we examined a pool of genes in which expression was upregulated after induction of demethylation by 5-Aza-2′-deoxycytidine (Aza) in the MCF-7 breast cancer cell line. Genome-wide demethylation was confirmed by monitoring the demethylation of a previously known gene, *SULT1A1.* Overall, 210 and 213 genes were found to be upregulated and downregulated (fold change ≥ 2), respectively, in common in cells treated with 5 and 10 μM of Aza. Network analysis of these 423 genes with altered expression patterns identified the involvement of a cancer related network of genes that were heavily regulated by TNF-α in breast tumorigenesis. Our results suggest that epigenetic dysregulation of cellular processes relevant to TNF-α-dependent apoptosis may be intimately involved in tumorigenesis in MCF-7 cells.

INTRODUCTION

Methylation of CpG at the promoter has been shown to play a key role in regulating gene activity in cellular development, differentiation, apoptosis, and carcinogenesis (Kulis and Esteller, 2010; Liu et al., 2010; Maegawa et al., 2010). Indeed, many tumor suppressors and proto-oncogenes undergo activation or inactivation during carcinogenesis *via* altered methylation. RASSF1A, BRCA1, and 15-Hydroxyprostaglandin dehy-dogenase are tumor suppressors with CpGs that are frequently methylated in breast cancer (Matros et al., 2005; Wolf et al., 2006; Yan et al., 2003). Genome-wide screening of genes with altered methylation has been conducted in breast cancer tis-sues (Tommasi et al., 2009; Van der Auwera et al., 2010) and cell lines (Rodenhiser et al., 2008a) with the goal of identifying genes en masse. Through these approaches, novel tumor suppressors including HOXB13, HNF1B, and MOGAT2 have been newly identified. Systematic screening has made it possible to elucidate the network of genes that are epigenetically regulated in breast cancer. For example, Li et al. conducted an Illumina methylation array for ER+ and ER- tissues of breast cancer and found that the inflammatory response, connective tissue disorders pathway was overwhelming in the cancer (Li et al., 2010).

CpG islands are frequently found at or near the promoter, and methylation of the majority of CpGs has commonly been observed in inactivated genes (Shao et al., 2007). However, methylation in only a portion of the CpGs (Kim et al., 2010) or even a single site could have induced inactivation (Sohn et al., 2010). Even though methylation at the promoter has mainly been linked to gene inactivation, methylation in other regions such as introns or the 3′-UTR have also been shown to induce inactivation (Laux et al., 1999; Malumbres et al., 1999). The methylation of CpG is closely associated with the chromatin structure; therefore, demethylation alone could not have induced gene activation often. A methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (Aza) has been widely applied to induce demethylation of the methylated CpG (Xiong et al., 2009). For example, this compound was utilized to confirm the restoration of gene activity that was caused by methylation. In some cases, treatment with Trichostatin A, a histone deacetylase inhibitor, together with Aza induced a synergistic upregulation, implying that methylation and chromatin structure are tightly linked (Rivenbark et al., 2006). Aza can also be utilized to identify novel genes that are epigenetically regulated. Examples in breast cancer include APAF-1, CEACAM6 and ZC3HDC1, which were found to be upregulated after treatment with Aza against the MCF-7 cancer cell; therefore, their expression was further examined in cancer tissues (Rivenbark et al., 2006; Xiong et al., 2009).

Genome-wide approaches have been confined to monitoring the methylation status using techniques such as differential methylation hybridization (Tommasi et al., 2009) and genotyping-based sequencing methods such as the Illumina methylation array (Li et al., 2010). The genome-wide approach using Aza has an advantage in that it can directly identify a pool of

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Received September 2, 2011; revised November 18, 2011; accepted November 18, 2011; published online January 2, 2012

Keywords: 5-Aza-2′-deoxycytidine, breast cancer, genome-wide expression, MCF-7, TNF-α

genes that are inactivated by methylation. However, only a few cases have been reported in cancer, and no network analysis has been conducted to the best knowledge of the authors.

In this study, we induced global demethylation in MCF-7 cells by Aza and conducted expression array analysis using a system harboring 30,968 genes. For 423 genes showing statistical significance, Ingenuity pathway analysis was conducted to reveal pivotal pathways that are epigenetically regulated. A cancer pathway in which TNF-α plays the central role was at the top pathway.

MATERIALS AND METHODS

Cell culture and 5-Aza-2′-deoxycytidine treatment

A human breast cancer cell line, MCF7, was purchased from the American Type Culture Collection (ATCC; USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Demethylation of the cytosine residues was achieved by exposing the cell to culture media containing a methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (Aza) (Sigma, USA), at 5 and 10 μM for 72 h. Culture medium with or without treatment was changed every 24 h.

Bisulfite sequencing

Chromosomal DNA extracted from the tissues was subjected to bisulfite treatment using an EpiTect Bisulfite kit (Qiagen, USA). The bisulfite-treated DNA was subjected to poly-merase chain reaction (PCR) to amplify the 372-bp promoter region of *SULT1A1* (nucleotides -503 to -131 of GenBank accession no. AB062428; transcriptional start site, +1) containing six CpG sites. The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The resulting products were purified using a Qiaex II gel extraction kit (Qiagen) and then sub-cloned into the pGEM-T vector. The DNA sequences were confirmed by analyzing each plasmid clone in both directions.

Methylation-specific PCR (MSP)

Chromosomal DNA was isolated from the cell culture in a 75 cm^2 culture flask using a genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. The extracted DNA was then eluted with 250 μl of distilled water. Sodium bisulfite modification of genomic DNA was conducted using an EpiTect Bisulfite kit (Qiagen) according to the manufacturer's protocol using 0.1 mg of purified DNA. PCR primers for the selected genes (Supplementary Table S1) were designed using the Methprimer program (http://www.urogene.org/methprimer/index1.html). One primer set was specific for amplification of methylated DNA and one was designed for amplification of unmethylated DNA. Quantitative PCR was conducted using a Power SYBR Green Kit (Applied Biosystems, USA) according to the protocols of the manufacturer. To assign a quantitative measure to the level of methylation, a methylation index was calculated for each sample using the following formula: methylation index = $[1 / (1 + 2^{-(CTu - CTme)})] \times 100\%$, as pre-viously described (Lu et al., 2007), where CTu is the average cycle threshold (CT) obtained from duplicate quantitative PCR analyses using the unmethylated primer set and CTme is the average CT obtained using the methylated primer pair.

Real time RT-PCR

Isolation of Total RNA from cell culture and reverse transcription were conducted as described previously (Kim et al., 2010). Expression levels of TNF-α was measured by real-time quantitative RT-PCR analysis. Duplicate reactions were performed for each sample using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, USA) with TNF-α-specific primers on an ABI 7300 instrument (Applied Biosystems). RNA quantity was normalized to GAPDH content, and gene expression was quantified according to the $2^{-\Delta Ct}$ method.

Microarray analysis

Approximately 5×10^6 proliferating MCF-7 cells treated with or without Aza were harvested and total RNA was extracted using an RNeasy mini kit (Qiagen). The RNA was then used for microarray analysis in duplicate using a Phalanx Human OneArray chip (Digital-Genomics, Korea) containing 30,968 human cDNA samples. Differential expression values, calculated as +Cy3/Cy5, where Cy3Signal > Cy5Signal, or -Cy3/Cy5, where Cy3Signal < Cy5Signal, were compared between the duplicate experiments. Clones differentially regulated in both experiments that had a significant ratio of Cy3 to Cy5 (defined as > 2) were selected for further analysis. All array data have been uploaded to the Gene Expression Omnibus (GEO) database, and can be accessed via their website (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE31441).

Pathway analysis

To identify pathways displaying methylation-specific altered expression patterns with potential roles in breast carcinogenesis, functional categorization and pathway construction were conducted using the Ingenuity Pathway Analysis (IPA) software tool produced by Ingenuity Systems. IPA utilizes an extensive database of functional interactions that are drawn from peer-reviewed publications and are manually maintained (Calvano et al., 2005). P-values for individual networks were obtained by comparing the likelihood of obtaining the same number of transcripts or greater in a random gene set as are actually present in the input file (i.e., the set of genes differentially expressed in the Aza treated MCF-7 cells) using Fischer's exact test based on the hypergeometric distribution. The highest confidence functional network was designated as the top network.

Statistical analysis

The heatmap data were obtained using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). A pool of genes that fit the top IPA network was submitted to the program. In the microarray analysis, observations with adjusted p-values equal to or greater than 0.05 were removed and thus precluded from further analysis. Following adjustment, remaining genes were defined as differentially expressed if they displayed at least a two-fold difference in expression levels between the control and Aza-treated groups to further reduce the number of false positive observations, and to enrich for biologically relevant expression changes. A student's *t*-test was used to detect differences in the mean levels of methylation for selected genes, as well as the expression level between the control and Aza-treated cells. Statistical analyses were conducted using SPSS for Windows, release 17.0 (SPSS Inc., USA). A $p < 0.05$ was considered to be statistically significant.

RESULTS

Aza induced genome-wide demethylation in MCF-7 cells

To induce genome-wide demethylation of the MCF-7 cells, they were treated with a methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (Aza), at 5 and 10 μM for 72 h. The efficacy of Aza was then monitored by measuring the methylation status of CpGs at the promoter of *SULT1A1*, which was previously iden-

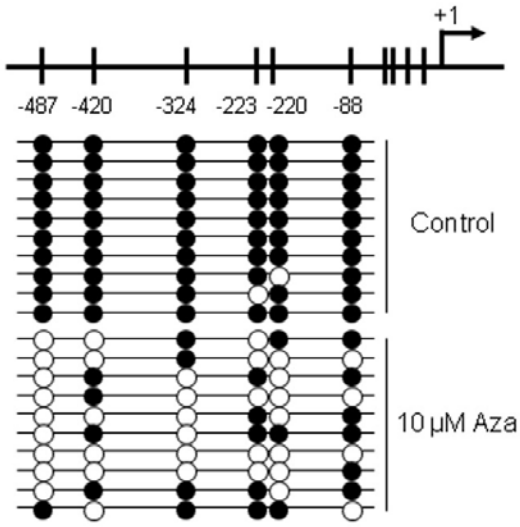

Fig. 1. Induction of demethylation by Aza in MCF-7 cells. The methylation status of CpG sites at the *SULT1A1* promoter was determined by sequencing after subcloning of the bisulfate-PCR product into the pGEM-T plasmid. Schematic diagram of the CpG sites at the promoter is drawn on the top. Methylated and unmethylated CpGs are indicated as black and open circles, respectively.

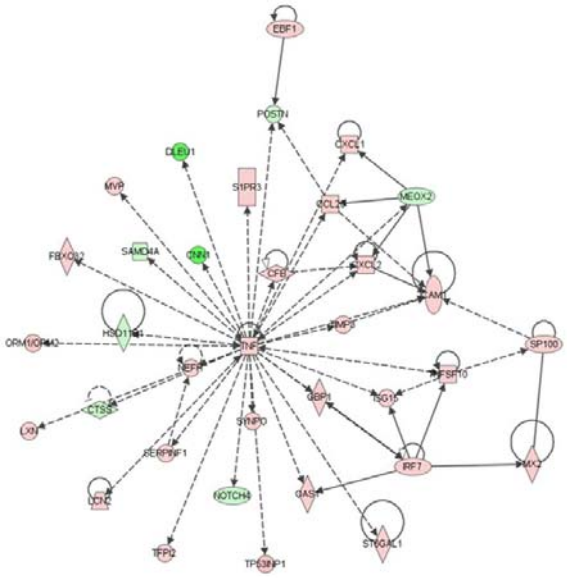

Fig. 2. Highest confidence network of genes displaying altered expression levels in response to demethylation in MCF-7 cells. According to IPA, the network is relevant to 'Cellular Movement, Cellular Development, Cancer'. Genes that were upregulated in the Aza-treated MCF-7 cells are red, while those that were downregulated are green, and the intensity of the color reflects the magnitude of expression change. Each interaction is supported by at least one literature reference, with solid lines representing direct interactions, and dashed lines representing indirect interactions.

tified as a breast cancer methylation marker (Kwon et al., 2006). To accomplish this, 372 bp of the promoter region containing six CpGs was amplified by PCR after bisulfite treatment of the chromosomal DNA. The PCR product was then subcloned into the pGEM-T plasmid and the methylation status of ten clones from the 10 μM Aza treated samples were determined. Overall, the methylation level of the Aza-treated cells was 38%, while the control cells showed 96% methylation (Fig. 1). These results implied that Aza successfully induced global hypomethylation in the MCF-7 cells.

TNF-α mediated pathways are involved in the network of methylation-altered genes

Genome-wide expression analysis was conducted using the RNA as probes isolated from MCF-7 cells treated with Aza. Overall, 577 genes and 846 genes from cells treated with 5 μM and 10 μM of Aza were found to be upregulated, while 967 genes and 1180 genes were found to be downregulated, respectively, with a cutoff of a two-fold change in expression. Overall, 210 upregulated and 213 downregulated genes fit the significance criteria and were observed in both Aza treatments. Top regulated genes were listed in Table 1. All of these genes were examined for functional interrelatedness using the Ingenuity Pathway Analysis software tool. The highest functional network (Score = 61) resulting from differential gene expression was designated as "Cellular Movement, Cellular Development, Cancer" (Figs. 2 and 3). Interestingly, TNF-α, the key extrinsic mediator of the TNF-induced model of cellular apoptosis, was most prominent in the network and appeared to function as a master signaling molecule of a network of all other gene transcripts potentially relevant to breast tumor development and progression. To examine whether the alteration of methylation and expression of TNF-α is involved in the breast tumorigenesis, they were monitored in the normal MCF-10A breast cell and the cancerous MCF-7 cell (Fig. 4). The methylation was low and the expression was upregulated in MCF-10A. These patterns are reminiscent of those in the Aza-treated MCF-7 cell, implying treatment of Aza to the cancer cell mimicked the normal cell. Cluster analysis of the 423 genes included in the top two networks is shown in Fig. 5.

The transcripts displaying the highest levels of altered expression within this network were *TIMP3* (4.6-fold increase in 5 μM Aza treated cells and 4.4-fold increase in 10 μM Aza treated cells) and *TP53INP1* (4.0-fold increase in 5 μM treated cells and 6.9-fold increase in 10 μM Aza treated cells) (Table 1). *TIMP3* has been shown to inhibit tumor growth, invasion and angiogenesis, and has previously been established as a hypermethylated and downregulated gene in breast cancer (Lui et al., 2005). *TP53INP1*, which plays a role in both cell cycle arrest and p53-mediated apoptosis (Weng et al., 2011), has been shown to be decreased in melanoma and its reduced expression was correlated with hypermethylation of a promoter (Bonazzi et al., 2009). Notable cancer-related genes within the TNF-α-dependent apoptosis pathways that were upregulated in the Aza-treated MCF-7 cells included *TNFSF10* (2.1-fold increase in both 5 and 10 μM Aza treated cells) and *ICAM1* (2.3-fold increase in both 5 and 10 μM Aza treated cells). *TNFSF10,* which is known to be a TNF-related apoptosis-inducing ligand, has been found to induce apoptosis in cancer cells, and its receptors (DcR1 and DcR2) had an aberrant methylation pattern in breast cancer (Shivapurkar et al., 2004). *ICAM1* has been shown to play a role as a cell adhesion molecule and is related to immune responses. TNF-α has been found to reinduce expression of *ICAM1*, which was downregulated in breast cancer (Budinsky et al., 1997). Significantly downregulated cancer-relevant genes included *POSTN* (3.0-fold decrease in both 5 and 10 μM Aza treated cells) and *NOTCH4* (3.0-fold decrease in 5 μM Aza treated cells and 5.0-fold decrease in 10 μM Aza treated cells). *POSTN* has been shown to be overexpressed in

Table 1. Genes in top network displaying differential expression after treatment of the MCF-7 cells with Aza

Symbol	Accession	Description	Fold change	
			5 μM Aza	10 μM Aza
CCL20	NM_004591.1	Chemokine (C-C motif) ligand 20	2.73	2.41
CFB	NM_001710.3	Complement factor B	3.07	2.53
CNN1	NM_001299.3	Calponin 1, basic, smooth muscle	-6.00	-10.0
CTSS	NM_004079.3	Cathepsin S	-2.50	-2.25
CXCL1	NM_001511.1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	2.44	3.33
CXCL2	NM_002089.1	Chemokine (C-X-C motif) ligand 2	2.28	2.19
DLEU1	AW303167	Deleted in lymphocytic leukemia, 1	-2.25	-10.0
EBF1	CR627349	Early B-cell factor 1	2.50	2.60
FBXO32	NM_058229.2	F-box protein 32	2.02	2.62
GBP1	NM_018284.1	Guanylate binding protein 1, interferon-inducible, 67 kDa	2.34	2.83
HSD11B1	NM_005525.2	Hydroxysteroid (11-beta) dehydrogenase 1	-3.50	-2.25
ICAM1	NM_000201.1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	2.26	2.26
IRF7	NM_004029.1	Interferon regulatory factor 7	2.01	2.21
ISG15	NM_005101.1	ISG15 ubiquitin-like modifier	2.28	2.42
LCN2	NM_005564.2	Lipocalin 2 (oncogene 24p3)	4.29	2.71
LXN	NM_020169.2	Latexin	3.21	2.61
MEOX2	NM_005924.3	Mesenchyme homeo box 2 (growth arrest-specific homeo box)	-2.67	-2.50
MVP	NM_005115.3	Major vault protein	2.50	2.54
MX2	NM_002463.1	Myxovirus (influenza virus) resistance 2 (mouse)	2.32	2.72
NEFH	NM_021076.2	Neurofilament, heavy polypeptide 200 kDa	2.22	2.20
NOTCH4	NM_004557.3	Notch homolog 4 (Drosophila)	-3.00	-5.00
OAS1	NM_002534.1	2′,5′-oligoadenylate synthetase 1, 40/46 kDa	3.47	2.56
ORM1/ORM2	NM_000607.1	Orosomucoid 1	5.56	4.52
POSTN	NM_006475.1	Periostin, osteoblast specific factor	-3.00	-3.00
S1PR3	NM_001001938.1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	2.66	2.05
SAMD4A	BX092102	Sterile alpha motif domain containing 4A	-2.50	-2.50
SERPINF1	NM_002615.4	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	3.34	2.95
SP100	AK160379	SP100 nuclear antigen	2.86	2.43
ST6GAL1	NM_173216.1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	4.60	2.87
SYNPO	AB028952	Synaptopodin	2.91	2.76
TFPI2	NM_006528.2	Tissue factor pathway inhibitor 2	2.01	2.82
TIMP3	NM_000362.3	Tissue inhibitor of metalloprotei--se 3 (Sorsby fundus dystrophy, pseudoinflammatory)	4.60	4.41
TNF	NM_000594.2	Tumor necrosis factor (TNF superfamily, member 2)	2.08	2.75
TNFSF10	NM_003810.2	Tumor necrosis factor (ligand) superfamily, member 10	2.07	2.13
TP53INP1	NM_033285.2	Tumor protein p53 inducible nuclear protein 1	3.99	6.85

breast cancer (Zhang et al., 2010) and is included in pathways to promote cancer cell survival, epithelial-mesenchymal transition (EMT), invasion, and metastasis (Ruan et al., 2009). Expression of *NOTCH4* was found to lead to tumor formation in mammary glands (Politi et al., 2004) and its signaling was shown to be induced by TNF (Ando et al., 2003).

Genes in the TNF-α mediated pathway are frequently hypermethylated

To determine if genes in the pathways displayed consistency between mRNA levels and DNA methylation in the Aza-treated MCF-7 cells, ten genes were randomly selected and their methylation status was examined by MSP analysis. Seven of these were upregulated genes and three were downregulated genes. Four (*ICAM1, TIMP3, TNF* and *TNFSF10*) were previously established as methylation markers in cancer or other diseases, while six (*CNN1, HSD11B1, ISG15, NOTCH4, SERPINF1,* and *S1RP3*) were novel genes for which the methylation status is not known. As shown in Fig. 6, six of the seven upregulated genes showed hypomethylation in the Aza-treated MCF-7 cells in a concentration-dependent manner. The six downregulated genes did not show any consistent methylation profile.

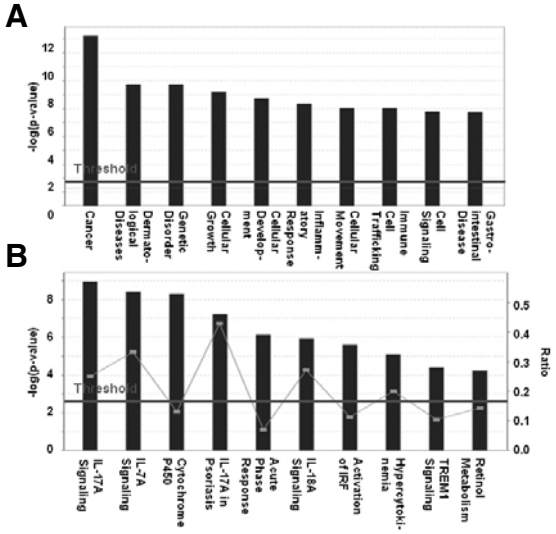

Fig. 3. Pathways most strongly associated with the significantly altered genes in the Aza-treated MCF-7 cells. (A) Top functional categories. (B) Canonical pathways. The Ingenuity software assigns a *P*-value based on the likelihood of obtaining the observed number of category or pathway-related molecules in a given data set by chance alone. The threshold line denotes the $P = 0.05$ level. The line graph represents the ratio of affected genes to the total number of genes in a pathway.

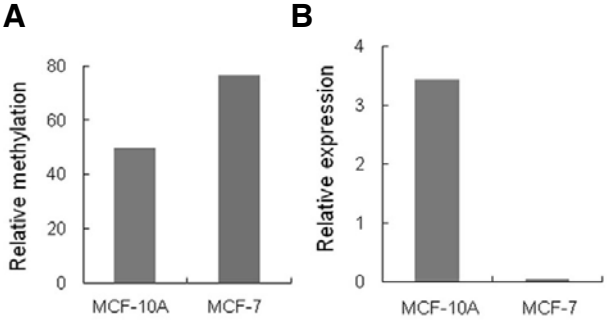

Fig. 4. Differential methylation and expression of TNF-α in normal and cancer cell lines. Real-time MSP (A) and real-time RT-PCR analysis (B) of TNF-α were performed in the normal cell line, MCF-10A, and the cancer cell line, MCF-7. Each sample was examined in duplicate and the average relative methylation and expression levels are presented.

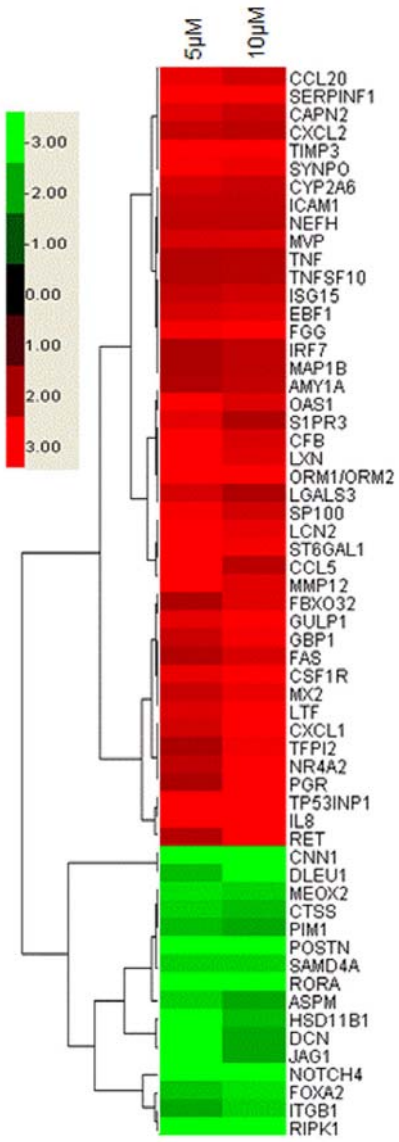

Fig. 5. Gene expression profiles of genes with altered expression in response to treatment of the MCF-7 cells with Aza. Heatmap analysis was conducted for the 59 genes that were included in the top two networks of IPA pathway analysis. Each row represents a gene, and each column represents the MCF-7 cells treated with 5 or 10 μM of Aza. As shown in the color bar, black represents no change, red represents upregulation, and green represents downregulation of gene expression.

DISCUSSION

This study was carried out to screen differentially expressed genes conferred by CpG methylation and thereby elucidate essential regulatory pathways in MCF-7 cells. 210 upregulated and 213 downregulated genes were observed in common from 5 μM and 10 μM Aza-treated cells. These relatively small numbers of genes are possibly due to the fact that Aza not only induces the re-expression of silenced genes through the demethylation of CpG islands, but it increases the expression of unmethylated genes. The agent also induces cell stress by DNA damage (Xiong and Epstein, 2009).

It is notable that the TNF-α pathway, which was most prominent in the MCF-7 cells, also ranks among the top networks in other breast cancer cells and other cancer tissues. For example, in a metastatic variant of the breast cancer cell line, MDA-MB-468LN, the genome-wide methylation profile identified the TNF-pathway as the second highest network (Chambers and Goss, 2008). In the case of prostate cancer, the TNF-α pathway was the top network in a genome-wide methyaltion profile (Kim et al., 2011). TNF-α, a pleiotropic cytokine whose expression has been detected in breast tissue, is the key mediator of TNF-α-dependent apoptosis pathways, and has been found to both contribute to and protect against breast tumorigenesis (Yin et al., 2009). The TNF-α gene promoter frequently displayed DNA methylation in T-cell lymphoma (Zhang et al., 2009). In addition, the cytokine is involved in apoptosis, cell survival, inflammation,

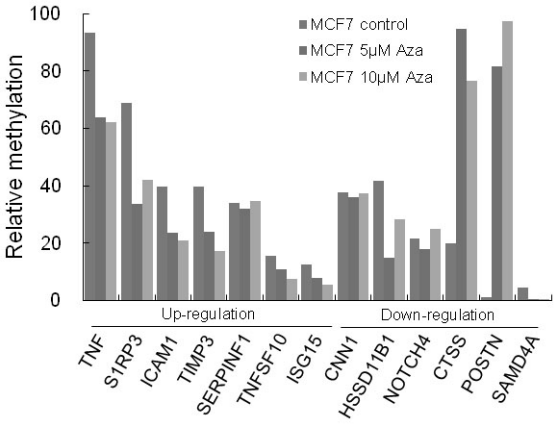

Fig. 6. MSP analysis of selected genes displaying altered expression in response to demethylation in the MCF-7 cells. Seven of the upregulated and six of the downregulated genes were randomly selected from the top network of IPA pathway and their methylation was examined by methylation-specific PCR. Each sample was analyzed in duplicate and the average relative methylation level is presented.

and immunity, and acts *via* two receptors (Dempsey et al., 2003; van Horssen et al., 2006). Its apoptotic activity is mediated through activation of death domain proteins such as TRADD and FADD, and caspases. For cell proliferation, TNF-α signals to Fos/Jun through the MAPK and JNK pathways, or to the NF-κB pathway. Our pathway analysis indicated that all three TNF-α-mediated apoptosis-relevant pathways (the MAPK pathway, NF-κB pathway, and the TNF-α-induced caspase-dependent death signaling pathway) are epigenetically regulated in MCF-7 cells, MDA-MB-468LN cells and prostate cancer. This fact implies that there is a precise signaling mechanism that determines the cellular fate as either proliferation or death.

It should be emphasized that the composites of interacting proteins with TNF-α were somewhat different, even though their pathways all included TNF-α as the core regulatory element in various tissues and the pathways contained all three TNF-α-related pathways. This may imply that TNF-α is linked to different networks depending on the cancer type. In fact, in MDA-MB-468LN cells, genes involved in chromatin remodeling such as ADNP, which is an element of the SWI/SNF chromatin remodeling complex, were uniquely involved (Rodenhiser et al., 2008b). In case of the prostate cancer, BIK and BAK1, which are BCL-2 antagonists, comprised the central elements (Kim et al., 2011). In the present study of MCF-7 cells, ICAM1 and TNFSF10 uniquely appeared in the network. These findings imply that TNF-α is linked to different networks in different cancer tissues, and even to different subtypes in single types of cancer tissue.

It should be noted that our study was limited in that the epigenetic regulation was based on just one cell type. Extending the examination further to various cell types and tissues might provide insight into common and/or pivotal TNF-α-related pathways. It should be noted that the epigenetic dysregulation could be caused by events other than changes in methyaltion such as histone modification. Co-treatment of the cell together with a histone acetylation inhibitor, Tricostatin A, might overcome the gene silencing effect that occurred when only Aza was used.

A key concern of our study was whether Aza might induce altered expression in a way other than methylation, thus spoiling the pathway analysis. Nevertheless, we have successfully shown that the majority of genes in the pathway underwent demethylation by confirming that eight of ten selected genes in the TNF-α pathway were appropriately demethylated.

In summary, we present here a genome-wide expression profile of MCF-7 breast cancer cells, which was compiled based on promoter methylation and the most comprehensive pathway assignment method currently available. Our findings lend credence to the strong link between epigenetic dysregulation of important cancer-relevant molecules, including those pertinent to TNF-α-dependent apoptosis. Furthermore, our findings indicate that the relevant alteration of expression of these molecules may be involved in breast carcinogenesis. Moreover, tumor-specific alterations in methylation patterns were shown to have the potential to serve as prognostic biomarkers in breast cancer development and progression. Further investigations into the mechanisms leading to the differential methylation observed in the present study, as well as associations of methylation status with breast tumor stage and progression may provide additional insights that could prove useful in estimating prognosis and determining treatment options.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This study was supported by the National Research Foundation of Korea (NRF) Grant (NRF-2011-C00056) and by the National Science Foundation of China (NSFC) Grant (30800891 & 81111140396).

REFERENCES

Ando, K., Kanazawa, S., Tetsuka, T., Ohta, S., Jiang, X., Tada, T., Kobayashi, M., Matsui, N., and Okamoto, T. (2003). Induction of Notch signaling by tumor necrosis factor in rheumatoid synovial fibroblasts. Oncogene *22,* 7796-7803.

Bonazzi, V.F., Irwin, D., and Hayward, N.K. (2009). Identification of candidate tumor suppressor genes inactivated by promoter methylation in melanoma. Genes Chromosomes Cancer *48,* 10-21.

Budinsky, A.C., Brodowicz, T., Wiltschke, C., Czerwenka, K., Michl, I., Krainer, M., and Zielinski, C.C. (1997). Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast-cancer cells *in vitro.* Int. J. Cancer *71,* 1086-1090.

Calvano, S.E., Xiao, W., Richards, D.R., Felciano, R.M., Baker, H.V., Cho, R.J., Chen, R.O., Brownstein, B.H., Cobb, J.P., Tschoeke, S.K., et al. (2005). A network-based analysis of systemic inflammation in humans. Nature *437,* 1032-1037.

Chambers, A.F., and Goss, P.E. (2008). Putative growth characteristics of micrometastatic breast cancer. Breast Cancer Res. *10,* 114.

Dempsey, P.W., Doyle, S.E., He, J.Q., and Cheng, G. (2003). The signaling adaptors and pathways activated by TNF superfamily. Cytokine Growth Factor Rev. *14,* 193-209.

Kim, T.W., Kim, Y.J., Lee, H.J., Min, S.Y., Kang, H.S., and Kim, S.J. (2010). Hs.137007 is a novel epigenetic marker hypermethylated and up-regulated in breast cancer. Int. J. Oncol. *36,* 1105-1111.

Kim, S.J., Kelly, W.K., Fu, A., Haines, K., Hoffman, A., Zheng, T., and Zhu, Y. (2011). Genome-wide methylation analysis identifies involvement of TNF-α mediated cancer pathways in prostate cancer. Cancer Lett. *302,* 47-53.

Kulis, M., and Esteller, M. (2010). DNA methylation and cancer. Adv. Genet. *70,* 27-56.

Kwon, M.S., Kim, S.J., Lee, S.Y., Jeong, J.H., Lee, E.S., and Kang, H.S. (2006). Epigenetic silencing of the sulfotransferase 1A1 gene by hypermethylation in breast tissue. Oncol. Rep. *15,* 27-32.

Laux, D.E., Curran, E.M., Welshons, W.V., Lubahn, D.B., and Huang, T.H. (1999). Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas. Breast

Cancer Res. Treat. *56,* 35-43.
Li, L., Lee, K.M., Han, W., Choi, J.Y., Lee, J.Y., Kang, G.H., Park, S.K., Noh, D.Y., Yoo, K.Y., and Kang, D. (2010). Estrogen and progesterone receptor status affect genome-wide DNA methylation profile in breast cancer. Hum. Mol. Genet. *19,* 4273-4277.
Liu, B., Tahk, S., Yee, K.M., Fan, G., and Shuai, K. (2010). The ligase PIAS1 restricts natural regulatory T cell differentiation by epigenetic repression. Science *330,* 521-525.
Lu, L., Katsaros, D., de la Longrais, I.A., Sochirca, O., and Yu, H. (2007). Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res. *67,* 10117-10122.
Lui, E.L., Loo, W.T., Zhu, L., Cheung, M.N., and Chow, L.W. (2005). DNA hypermethylation of TIMP3 gene in invasive breast ductal carcinoma. Biomed. Pharmacother. *59 Suppl. 2,* S363-365.
Maegawa, S., Hinkal, G., Kim, H.S., Shen, L., Zhang, L., Zhang, J., Zhang, N., Liang, S., Donehower, L.A., and Issa, J.P. (2010). Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res. *20,* 332-340.
Malumbres, M., Pérez de Castro, I., Santos, J., Fernández Piqueras, J., and Pellicer, A. (1999). Hypermethylation of the cell cycle inhibitor p15INK4b 3′-untranslated region interferes with its transcriptional regulation in primary lymphomas. Oncogene *18,* 385-396.
Matros, E., Wang, Z.C., Lodeiro, G., Miron, A., Iglehart, J.D., and Richardson, A.L. (2005). BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. Breast Cancer Res. Treat. *91,* 179-186.
Politi, K., Feirt, N., and Kitajewski, J. (2004). Notch in mammary gland development and breast cancer. Semin. Cancer Biol. *14,* 341-347.
Rivenbark, A.G., Jones, W.D., Risher, J.D., and Coleman, W.B. (2006). DNA methylation-dependent epigenetic regulation of gene expression in MCF-7 breast cancer cells. Epigenetics *1,* 32-44.
Rodenhiser, D., Andrews, J., Kennette, W., Sadikovic, B., Mendlowitz, A., Tuck, A., and Chambers, A. (2008a). Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. Breast Cancer Res. *10,* R62.
Rodenhiser, D.I., Andrews, J., Kennette, W., Sadikovic, B., Mendlowitz, A., Tuck, A.B., and Chambers, A.F. (2008b). Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. Breast Cancer Res. *10,* R62.
Ruan, K., Bao, S., and Ouyang, G. (2009). The multifaceted role of periostin in tumorigenesis. Cell Mol. Life Sci. *66,* 2219-2230.
Shao, L., Cui, Y., Li, H., Liu, Y., Zhao, H., Wang, Y., Zhang, Y., Ng, K.M., Han, W., Ma, D., et al. (2007). CMTM5 exhibits tumor suppressor activities and is frequently silenced by methylation in carcinoma cell lines. Clin. Cancer Res. *13,* 5756-5762.
Shivapurkar, N., Toyooka, S., Toyooka, K.O., Reddy, J., Miyajima, K., Suzuki, M., Shigematsu, H., Takahashi, T., Parikh, G., Pass, H.I., et al. (2004). Aberrant methylation of trail decoy receptor genes is frequent in multiple tumor types. Int. J. Cancer *109,* 786-792.
Sohn, B.H., Park, I.Y., Lee, J.J., Yang, S.J., Jang, Y.J., Park, K.C., Kim, D.J., Lee, D.C., Sohn, H.A., Kim, T.W., et al. (2010). Functional switching of TGF-beta1 signaling in liver cancer via epigenetic modulation of a single CpG site in TTP promoter. Gastroenterology *138*, 1898-1908.
Tommasi, S., Karm, D.L., Wu, X., Yen, Y., and Pfeifer, G.P. (2009). Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer. Breast Cancer Res. *11*, R14.
Van der Auwera, I., Yu, W., Suo, L., Van Neste, L., van Dam, P., Van Marck, E.A., Pauwels, P., Vermeulen, P.B., Dirix, L.Y., and Van Laere, S.J. (2010). Array-based DNA methylation profiling for breast cancer subtype discrimination. PLoS One *5,* e12616.
van Horssen, R., Ten Hagen, T.L., and Eggermont, A.M. (2006). TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist *11,* 397-408.
Weng, W., Yang, Q., Huang, M., Qiao, Y., Xie, Y., Yu, Y., jing, A., and Li, Z. (2011). c-Myc inhibits TP53INP1 expression via promoter methylation in esophageal carcinoma. Biochem. Biophys. Res. Commun. *405,* 278-284.
Wolf, I., O'Kelly, J., Rubinek, T., Tong, M., Nguyen, A., Lin, B., Tai, H., Karlan, B., and Koeffler, H. (2006). 15-hydroxyprostaglandin dehydrogenase is a tumor suppressor of human breast cancer. Cancer Res. *66,* 7818-7823.
Xiong, J., and Epstein, R.J. (2009). Growth inhibition of human cancer cells by 5-aza-2′-deoxycytidine does not correlate with its effects on INK4a/ARF expression or initial promoter methylation status. Mol. Cancer Ther. *8,* 779-785.
Xiong, H., Qiu, H., Zhuang, L., Jiang, R., and Chen, Y. (2009). Effects of 5-Aza-CdR on the proliferation of human breast cancer cell line MCF-7 and on the expression of Apaf-1 gene. J. Huazhong Univ. Sci. Technolog. Med. Sci. *29,* 498-502.
Yan, P.S., Shi, H., Rahmatpanah, F., Hsiau, T.H., Hsiau, A.H., Leu, Y.W., Liu, J.C., and Huang, T.H. (2003). Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res. *63,* 6178-6186.
Yin, Y., Chen, X., and Shu, Y. (2009). Gene expression of the invasive phenotype of TNF-alpha-treated MCF-7 cells. Biomed. Pharmacother. *63,* 421-428.
Zhang, Q., Wang, H.Y., Bhutani, G., Liu, X., Paessler, M., Tobias, J.W., Baldwin, D., Swaminathan, K., Milone, M.C. and Wasik, M.A., (2009). Lack of TNFalpha expression protects anaplastic lymphoma kinase-positive T-cell lymphoma (ALK+ TCL) cells from apoptosis. Proc. Natl. Acad. Sci. USA *106,* 15843-15848.
Zhang, Y., Zhang, G., Li, J., Tao, Q., and Tang, W. (2010). The expression analysis of periostin in human breast cancer. J. Surg. Res. *160,* 102-106.