Gene Expression Analysis in Human Gastric Cancer Cell Line Treated with Trichostatin A and *S*-Adenosyl-L-homocysteine Using cDNA Microarray

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Trichostatin A (TSA) and *S*-adenosyl-L-homocysteine (AdoHcy) have been reported to affect histone modifications. To investigate the effects of two drugs that can reportedly affect chromatin remodeling, we analyzed the gene expression profiles of TSA and AdoHcy in a gastric cancer cell line using 14 K cDNA microarray. The significant analysis of microarray (SAM) identified 98 and 43 differentially expressed genes in TSA and AdoHcy treated sets, respectively, and selected genes were functionally classified. In the gastric cancer cell line, genes related to cell communication, cell growth/maintenance, and morphogenesis were highly expressed with TSA, and genes with cell growth/maintenance, metabolism, oxidoreductase activity were upregulated with AdoHcy. Genes downregulated with TSA included those controlling the cell cycle, cell growth/proliferation, DNA binding, and metabolism, whereas genes involved in calcium signaling, cell growth/proliferation, and metabolism were downregulated with AdoHcy. Furthermore, we identified the genes commonly expressed in both drug treatments. Compared to TSA, AdoHcy did not induce apoptosis in the SNU-16 gastric cancer cell line, and RT-PCR was performed for selective genes to confirm the microarray data. This gene expression profile analysis with TSA and AdoHcy should contribute to a greater understanding of the molecular mechanism of chromatin remodeling and cancer, and provide candidate genes for further studies involving the roles of histone modifications in gastric cancer.

Key words cDNA microarray; trichostatin A; *S*-adenosyl-L-homocysteine; gastric cancer

Gastric cancer is one of the most common malignancies and a leading cause of cancer-related death worldwide.[2,3)] Little is known about the genes associated with its diverse clinical properties, such as metastatic status, invasiveness, histological type, and responsiveness to chemotherapy.[4)] Although recent studies have shown many genetic alterations in gastric cancer, these are hardly sufficient to understand the carcinogenesis of this disease.

In eukaryotic transcription, chromatin modifications by histone acetyltransferase (HAT) or histone deacetyltransferase (HDAC) represent a fundamental mechanism of transcriptional regulation.[5)] Recent evidence suggests that histone acetylation alters nucleosomal structures and facilitates the accessibility of transcription related factors to chromatin DNA by the disruption of interactions between histones and DNA.[6—8)] As an HDAC inhibitor, trichostatin A (TSA) has been known to cause a variety of phenotypic changes, including cell-cycle arrest in the G1/G2 phase, apoptosis, and differentiation in cultured transformed cells.[9—11)]

Protein methylation is a post-translational modification that is involved in cytoplasmic signal transduction and cell differentiation. Recently, important progress has been reported about its roles in the nucleus. Methylation of histone tail lysines and arginines has been linked to transcriptional activation and repression.[12)] Protein methylation utilizes *S*-adenosyl-L-methionine as a methyl donor, converting it to *S*-adenosyl-L-homocysteine (AdoHcy), which in turn is a potent inhibitor of methylation reactions.[13)] AdoHcy potentiates tumor necrosis factor-induced cytotoxicity in human breast carcinoma cells, and is a competitive inhibitor of histone lysine methyltransferase in rat brain.[14,15)] A recent report has suggested that histone H3 lysine 9 methylation is inhibited by increasing concentrations of AdoHcy.[16)] Protein arginine methyltransferases such as PRMTs can be inhibited by another intermediate molecule in the methylation pathway, adenosine dialdehyde (AdOx).[17)]

The importance of balance between acetylation and methylation is very important in transcription.[18)] Moreover, physical and functional interactions between deacetylation (HDAC1) and methylation (SU*VAR*3-9) clearly indicate the close connection between histone deacetylation and methylation.[19)]

Fast advances in cDNA microarray technologies have enabled the detection of global gene expression changes. In this study, we used this technology using 14 K cDNA microarray for the detection of altered gene expression patterns in a gastric cancer cell line treated with TSA and AdoHcy, both of which are able to affect post-translational modifications including histone acetylation and methylation.

MATERIALS AND METHODS

Cell Cultures The gastric carcinoma cell line, SNU-16, was grown in RPMI 1640 supplemented with 10% FBS and antibiotics in 5% CO_2. The cells were grown to about 70—80% confluence and treated with 330 nM TSA (Sigma) and 0.2 mM AdoHcy (Sigma) for 24 h.

RNA Extraction and Hybridization Total RNA was isolated from drug-treated cells using RNeasy midi-prep (Qiagen). The construction of a fluorescence probe and hybridization were performed using a 3DNA array detection kit (Genisphere).

cDNA Microarrays cDNA microarrays were performed on a cDNA chip containing 14080 cDNA clones selected

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from the KUGI Unigenes (http://kugi.kribb.re.kr). Each slide contained 704 control genes to normalize the signal intensities of the different fluorescent dyes.

Imaging and Image Analysis Each hybridization array was scanned on a ScanArray 5000 (Gsi Lumonics). The intensity of each hybridization signal was calculated by the GenePix Pro 4.0 program (Axon). The significantly expressed genes were identified by one class SAM (Significant Analysis of Microarray) analysis. The identified genes were selected according to the following the criteria: the FDR (False Discovery Rate) was less than 0.15%, and the average fold-change greater than 1.8 from six repeated experiments.

Clustering Hierarchical clustering analysis was performed using a relative gene expression ratio (Cy5/Cy3) to examine the relatedness among expression patterns of genes between experimental and reference. The analysis was performed using the software programs "Cluster" and "TreeView".[20]

Annotation of Gene Functions The selected genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.nuaid.nih.gov/David/), which automatically classifies the genes into known function/pathway groups. Genes that remained unclassified were assigned manually based on information from the Stanford Online Universal Resource for Clones and Expressed sequence tags (SOURCE) (http://genomewww5.stanford.edu/cgi-bin/SMD/source/sourceSearch).

RT-PCR cDNA was synthesized with oligo(dT) and a SuperScript RT-PCR kit (Invitrogen). RT generated cDNA was amplified by PCR using gene-specific primers and Taq polymerase, as described in Table 4. β-Actin was used as an internal control.

Quantification of Cell Death Histone-associated DNA fragments released from nuclear fractions of TSA and AdoHcy treated cells were quantified using Cell Death Detection ELISA (Roche Diagnostics) according to the manufacturer's instructions. SNU-16 cells were treated with TSA (330 nM) and AdoHcy (0.2 mM) for 24 h at 37 °C. After cell lysis and centrifugation, the cytoplasmic fractions were pre-diluted to 1 : 5 with an incubation buffer and tested for nucleosome formation by ELISA.

RESULTS AND DISCUSSION

cDNA Microarray Analysis We analyzed differentially expressed genes in TSA and the AdoHcy treated human gastric cancer cell line (SNU-16) using a cDNA microarray containing 14080 (14 K) sequence verified cDNA genes on glass slides. Total RNAs were isolated from cells treated with both drugs, and labeled with Cy-5. Reference cDNA probes from untreated cells were labeled with Cy-3. To exclude labeling bias, each experiment was repeated six times. Genes showed more than a 1.5-fold change in more than three experiments and were present in more than 80% of all the experimental sets that were filtered. Microarray analysis obtained 7054 of 14080 genes from the TSA-treated cells and 2599 of 14080 genes from the AdoHcy-treated cells after filtering in the cluster analysis program. Hierarchical clusterings of gene expression in the TSA-, AdoHcy-treated and untreated cells are shown in Figs. 1A and B. Average log-transformed ratios of each gene which represents the average ratio value of each gene from the six experiments, were calculated, and the genes were ranked according to those values.

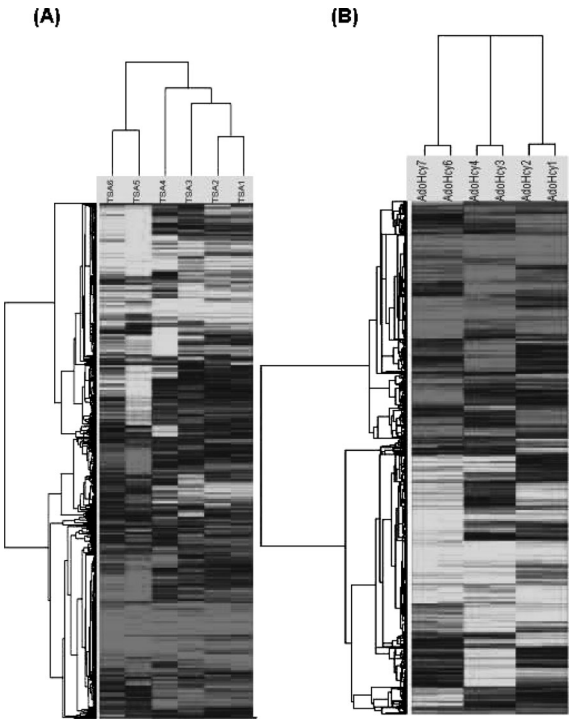

Fig. 1. Hierarchical Clustering Based on Gene Expression Data from TSA (A) and AdoHcy (B) Treated Gastric Cancer Cell Line

The dendrogram is based on 7054 (TSA) and 2599 (AdoHcy) genes that passed the filtering criteria. The hierarchical clustering analysis was generated using the programs "Clustering" and "Treeview".

We performed two statistical analyses of the filtered genes and compared their results to find the more significantly altered genes. First, average log-transformed ratios were obtained from the fold change method. Second, we applied a one class response type of SAM.[21] This analysis generated a list of genes with an average Cy-5/Cy-3 ratio significantly different from 1.0, along with an estimate of the percentage of such genes identified by chance or the FDR, which is based on permutations of repeated measurements. The genes with a threshold value of FDR less than 0.15% yielded 265 of 7054 genes that contained 186 positive and 79 negative significant genes from TSA-treated sets and 195 of 2599 genes that included 89 positive and 106 negative significant genes from the AdoHcy-treated set. To identify more significantly expressed genes from the two methods, we compared the genes obtained by the ranked data with the genes selected by the SAM analysis. Finally, we obtained 98 genes that contained 78 up- and 20 down-regulated genes from TSA-treated sets and 43 genes that included 21 up- and 22 down-regulated genes from AdoHcy-treated sets. These genes showed more than 1.8-fold change in average in the six experiments.

The Up-Regulated Genes by TSA By applying the SAM analysis, we first identified 186 genes out of 14080 genes that appear to be up-regulated by TSA treatment. Further analysis of the genes selected from the ranked data method with the SAM analysis selected 78 genes as significantly over-expressed by TSA treatment. To understand the biological significance of the genes, selected genes were cat-

Table 1. Functional Classification of Genes with Altered Expression in Response to TSA

Classification	Accession ID	Symbol	Gene name	Expression level (Log_2 ratio)[a)]
A. TSA up regulation				
Cell communication	NM_004363.1	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	1.05
	NM_005532.1	IFI27	interferon, alpha-inducible protein 27	1.36
	NM_003260.3	TLE2	transducin-like enhancer of splite 2 (E(sp1) homolog, Drosophila)	1.4
	NM_002610.3	PDK1	pyruvate dehydrogenase kinase, isoenzyme 1	1.03
	NM_006703.2	NUDT3	nudix(nucleoside diphosphate linked moiety X)-type motif 3	1.08
	NM_006788.2	RALBP1	ralA binding protein 1	1.17
	NM_004105.2	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1.28
Cell death	NM_000389.2	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.18
	NM_001048.2	SST	somatostatin	1.41
Cell growth and/or maintenance	NM_001759.2	CCND2	cyclin D2	1.14
	NM_182914.1	SYNE2	spectrin repeat containing, nuclear envelope 2	1.05
	NM_004877.1	GMFG	glia maturation factor, gamma	1.06
	NM_014865.1	CNAP1	chromosome condensation-related SMC-associated protein 1	0.99
	NM_002890.1	RASA1	RAS p21 protein activator (GTPase activating protein) 1	1.05
	NM_002658.1	PLAU	plasminogen activator, urokinase	1.34
	NM_007254.2	PNKP	polynucleotide kinase 3′-phosphatase	1.08
Metabolism	NM_006294.2	UQCRB	ubiquinol-cytochrome c reductase binding protein	1.33
	NM_019852.2	METTL3	methyltransferase like 3	1.28
	NM_002051.1	GATA3	GATA binding protein 3	1.24
	NM_021010.1	DEFA5	defensin, alpha 5, Paneth cell-specific	1.41
	NM_002774.2	KLK6	kallikrein 6 (neurosin, zyme)	1.16
	NM_017626.1	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	1.18
Morphogenesis	NM_000232.2	SGCB	sarcoglycan, beta (43 kDa dystrophin-associated glycoprotein)	1.14
	NM_012427.3	KLK5	kallikrein 5	0.97
	NM_000435.1	NOTCH3	Notch homolog 3 (drosophila)	1.15
Nucleotide binding	NM_003733.2	OASL	2′-5′-oligoadenylate synthetase-like	1.04
	NM_007350.1	PHLDA1	pleckstrin homology-like domain, family A, member 1	0.96
Protein binding	NM_015556.1	SIPA1L1	signal-induced proliferation-associated 1 like 1	1.14
	NM_016558.2	SCAND1	SCAN domain containing 1	2.09
	NM_001666.1	ARHGAP4	Rho GTPAse activating protein 4	1.06
	NM_005648.2	TCEB1	transcription elongation factor B(SIII), polypeptide 1 (15 kDa, elongin C)	1.13
	NM_006767.1	LZTR1	leucine-zipper-like transcriptional regulator. 1	1.08
Miscellaneous	NM_052985.1	WDR10	WD repeat domain 10	0.95
	NM_017902.1	HIF1AN	hypoxia-inducible factor 1, alpha subunit inhibitor	1.31
	NM_001970	EIF5A	eukaryotic translation initiation factor 5A	1.18
	NM_003666.2	BLZF1	Basic leucine zipper nuclear factor 1 (JEM-1)	1.09
	NM_003684.3	MKNK1	MAP kinase-interacting serine/threonine kinase 1	1.05
B. TSA down regulation				
Cell cycle	NM_004701.2	CCNB2	Cyclin B2	−1.49
	NM_198433.1	STK6	Serine/threonine kinase 6	−1.63
	NM_002417.2	MK167	antigen identified by monoclonal antibody Ki-67	−1.05
	NM_001255.1	CDC20	CDC20 cell division cycle 20 homolog (S.cerevisiae)	−1.37
Cell growth and/or maintenance	NM_020401.1	NUP107	Nuclear pore complex protein	−1.07
	NM_021230.1	MLL3	Myeloid/lymphoid or mixed-lineage leukemia 3	−1.18
	NM_005030.3	PLK	Polo-like kinase (Drosophila)	−1.63
Cell proliferation	NM_006600.2	NUDC	nuclear distribution gene C homolog (A. nidulans)	−1.02
	NM_004804.2	CIAO1	WD40 protein Ciao 1	−1.28
DNA binding	NM_002096.1	GTF2F1	general transcription factor IIF, polypeptide 1	−1.25
Metabolism	NM_002803.2	PSMC2	Proteasome (prosome, macropain) 26S subunit	−0.86
	NM_000505.2	F12	coagulation factor XII (Hageman factor)	−1.04
	NM_005163.1	AKT1	V-akt murine thymoma viral oncogene homolog 1	−0.99
	NM_003090.1	SNRPA1	Small nuclear ribonucleoprotein polypeptide A′	−1.01
Miscellaneous	NM_024545.2	SAP130	MSin3A-associated protein 130	−1.07
	NM_002375.2	MAP4	Microtubule-associated protein 4	−1.19

a) Expression level indicates the natural logarithm of the Cy5/Cy3 ratio of the expression value.

egorized into functional groups by the gene annotation program. As expected, by disrupting histone mediated transcriptional repression, genes whose functions are involved in various cellular activities are inducible by TSA (Table 1A). Functions that showed significant alterations of expression included cell communication, cell growth and/or maintenance, metabolism, and morphogenesis. Several of the known genes involved in the regulation of gene expression including, transcription factors and transcription related genes, were up-regulated by TSA. Transcription factor GATA-3 has its physiological function in the immune system, especially in T-cell survival and homing to secondary lymphoid organs.[22] Another transcription factor, hypoxia-inducible factor-1 (HIF-1), is essential for cellular adaptation to decreased oxygen availability, and its related gene, HIF-1 α subunit inhibitor, was upregulated in this study. It was no-

Table 2. Functional Classification of Genes with Altered Expression in Response to AdoHcy

Classification	Accession ID	Symbol	Gene name	Expression level (Log_2 ratio)[a)]
A. AdoHcy up regulation				
Cell communication	NM_003682.2	MADD	MAP-kinase activating death domain	1.46
Cell growth and/or maintenance	NM_016426.3	GTSE1	G-2 and S-phase expressed 1	1.27
	NM_003254.1	TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	1.06
	NM_006812.1	OS-9	amplified in osteosarcoma	1.1
	NM_001376.2	DNCH1	dynein, cytoplasmic, heavy polypeptide 1	1.35
Metabolism	NM_016035.1	COQ4	coenzyme Q4 homolog (yeast)	1.13
	NM_006556.2	PMVK	phosphomevalonate kinase	1.12
	NM_014595.1	NT5C	5′,3′-nucleotidase, cytosolic	1.08
	NM_006799.2	PRSS21	protease, serine, 21 (testisin)	1.45
Oxidoreductase activity	NM_023944.1	CYP4F12	Cytochrome P450, family 4, subfamily F, polypeptide 12	1.14
	NM-007103.2	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51 kDa	1.31
Protein binding	NM_003127.1	SPTAN1	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	1.21
	NM_004433.2	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	1.51
	NM_002167.2	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	0.98
Miscellaneous	NM_003297.1	NR2C1	nuclear receptor subfamily 2, group C, member 1	1.42
	NM_021009.2	UBC	ubiquitin C	1.02
	NM_003040.2	SLC4A2	Solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)	1.11
	NM_021971.1	GMPPB	GDP-mannose pyrophosphorylase B	1.02
	NM_012410.1	PSK-1	type I transmembrane receptor (seizure-related protein)	1.04
	—	—	1BO1 (H.sapiens) A Chain A, Phosphatidylinositol Phosphate Kinase Type II Beta	1.25
	NM_0.80861.3	SSB3	SPRY domain-containing SOCS box protein SSB-3	1.38
B. AdoHcy down regulation				
Calcium ion binding	NM_005184.1	CALM3	calmodulin 3 (phosphorylase kinase, delta)	−1.32
	NM_003127.1	SPTAN1	Spectrin, alpha, non-erythrocytic 1	−0.76
Cell growth and/or maintenance	NM_012242.1	DKK1	dickkopf homolog 1 (Xenopus laevis)	−0.63
	NM_003226.1	TFF3	trefoil factor 3 (intestinal)	−0.82
Cell proliferation	NM_004111.4	FEN1	Flap structure-specific endonuclease 1	−0.94
	NM_013400.1	REPIN1	replication initiator 1	−1.07
Metabolism	NM_004905.2	PRDX6	peroxiredoxin 6	−1.08
	XM_113678.4	NUP160	nucleoporin 160 kDa	−0.88
	NM_000858.3	GUK1	Guanylate kinase 1	−0.73
	NM_000386.2	BLMH	Bleomycin hydrolase	−1.41
	NM_020202.2	NIT2	Nit protein 2	−0.86
	NM_003133.1	SRP9	Signal recognition particle 9 kDa	−0.84
	NM_003145.2	SSR2	signal sequence receptor, beta (translocon)	−1.13
	NM_144563.1	RPIA	ribose 5-phosphate isomerase A	−0.96
Miscellaneous	NM_031434.2	C7orf21	chromosome 7 open reading frame 21	−0.94
	NM_174889.2	LOC91942	Hydrothetical protein LOC91942	−0.86
	XM_086257.2	—	similar to ribosomal protein S15	−0.7
	NM 078642	CypB	Cyclophilin B (peptidylproplyl isomerase B) mRNA	−1.31

a) Expression level indicates the natural logarithm of the Cy5/Cy3 ratio of the expression value.

table that some of the genes related to certain types of cancer through chromatin remodeling were upregulated with this treatment, too. As reported previously and mentioned above, as a cell death related gene, TSA induced cyclin-dependent kinase inhibitor p21.[23)] The growth arrest at the G1 phase by HDAC inhibitor is thought to be highly dependent on the up-regulation of p21/WAF1.

The Up-Regulated Genes by AdoHcy Compared to the TSA treatment, 21 genes were up-regulated with AdoHcy treatment, and these genes are functionally classified in Table 2A. This is the first demonstration of the expression patterns by the methyltransferase inhibitor AdoHcy-treated gastric cancer cell line using cDNA microarray. However, even though Wollwork *et al.* and Kim *et al.* reported that histone H3 and H4 lysine residue methylating enzyme and histone H3 lysine 9 methylation was inhibited by AdoHcy, respectively, it should be noted that AdoHcy affects other protein methyltransferases, too.[15,16)] According to the functional classification by annotation program, genes associated with cell communication, cell growth and/or maintenance, metabolism, oxidoreductase activity, and protein binding were up-regulated in AdoHcy treatment. The genes involved in oxidoreductase activity, such as cytochrome P450 and NADH dehydrogenase flavoprotein 1, were upregulated. Cytochrome P450, induced in this study, is a family of enzymes involved in the metabolism of many drugs and in the synthesis of steroid hormones. Also, MAP-kinase activating death domain (MADD) protein is involved in TNF-α induced activation of MAP-kinase.[24)]

The Down-Regulated Genes by TSA and AdoHcy Among those 20 genes repressed by TSA treatment are genes directly involved in the cell cycle or in regulating the cycle (Table 1B). Over-expression of B-type cyclins has been reported in various tumor types. Cyclins B2 could contribute to

Table 3. Functional Classification of Genes with Commonly Altered Expression in Response to Both TSA and AdoHcy

Classification	Accession ID	Symbol	Gene name	Expression level (Log_2 ratio)[a)]
A. TSA/AdoHcy up regulation				
Cell communication	NM_033133.3	CNP	2′,3′-cyclic nucleotide 3′ phosphodiesterase	0.82
Cell death	NM_002342.1	LTBR	lymphotoxin beta receptor (TNFR superfamily, member 3)	0.62
	NM_016426.3	GTSE1	G-2 and S-phase expressed 1	0.79
	NM_002087.1	GRN	granulin	0.79
	NM_004082.2	DCTN1	dynactin 1 (p150, glued homolog, Drosophila)	0.78
Kinase activity	NM_006314.1	CNK1	connector enhancer of KSR-like (Drosophila kinase suppressor of ras)	0.81
	NM_006556.2	PMVK	phosphomevalonate kinase	0.84
	NM_001291.1	CLK2	CDC-like kinase 2	0.83
Metabolism	NM_000456.1	SUOX	sulfite oxidase	0.65
	NM_016035.1	COQ4	coenzyme Q4 homolog (yeast)	0.78
	NM_006033.1	LIPG	lipase, endothelial	0.62
	NM_006799.2	PRSS21	protease, serine, 21 (testisin)	1.13
	NM_022366.1	TFB2M	transcription factor B2, mitochondrial	0.96
	NM_007103.2	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51 kDa	0.90
	NM_032717.3	MGC11324	hypothetical protein MGC11324	0.69
Metal ion binding	NM_003127.1	SPTAN1	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	
		PRSS3	protease, serine, 3 (mesotrypsin)	0.63
	NM_015062.3	PRC	PGC-1 related co-activator	0.69
	NM_013342.1	TFPT	TCF3 (E2A) fusion partner (in childhood Leukemia)	0.68
	NM_004433.2	ELF3	E74-like factor 3 (ets domain transcription factor, epithelialspecific)	0.98
Protein binding	NM_002167.2	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	0.65
	NM_024056.2	MGC5576	hypothetical protein MGC5576	0.62
			Homo sapiens, Similar to Rab GTPase-activating protein PRC17, clone IMAGE:5743752, mRNA	0.71
	NM_012256.2	ZNF212	zinc finger protein 212	0.62
	NM_003297.1	NR2C1	nuclear receptor subfamily 2, group C, member 1	1.20
Miscellaneous	NM_017765.1	FLJ20320	hypothetical protein FLJ20320	0.73
	NM_025257.1	C6orf29	chromosome 6 open reading frame 29	0.81
	NM_017739.1	FLJ20277	O-linked mannose beta1,2-*N*-acetylglucosaminyltransferase	1.21
	NM_018415.1	TRERF1	transcriptional regulating factor 1	0.67
	XM_209143.1	FLJ41131	FLJ41131 protein	0.93
	NM_024532.2	PF20	PF20	0.75
	NM_015528.1	DKFZP566H073	DKFZP566H073 protein	0.69
	XM_351694.1		similar to retinitis pigmentosa 9 homolog; Pim-1 associated protein	0.64
B. TSA/AdoHcy down regulation				
Cell communication	NM_005505.3	SCARB1	scavenger receptor class B, member 1	−0.62
	NM_000165.2	GJA1	gap junction protein, alpha 1, 43 kDa (connexin 43)	−0.65
	NM_020474.2	GALNT1	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide) *N*-acetylgalactosaminyl-transferase 1 (GalNAc-T1)	−0.70
Cell growth/ maintenance	NM_004656.2	BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	−0.64
	NM_006600.2	NUDC	nuclear distribution gene C homolog (A. nidulans)	−0.75
	NM_152333.1	C14orf69	chromosome 14 open reading frame 69	−0.72
	NM_024057.2	Nup37	nucleoporin Nup37	−0.63
Metabolism	NM_031280.2	MRPS15	mitochondrial ribosomal protein S15	−0.64
	NM_005891.1	ACAT2	acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase)	−0.66
	NM_004718.2	COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	−0.97
	NM_001216.1	CA9	carbonic anhydrase IX	−0.67
	NM_000386.2	BLMH	bleomycin hydrolase	−1.12
	NM_144563.1	RPIA	ribose 5-phosphate isomerase A (ribose 5-phosphate epimerase)	−0.78
	NM_004911.3	ERP70	protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)	−0.69
	NM_004930.1	CAPZB	capping protein (actin filament) muscle Z-line, beta	−0.61
	NM_005184.1	CALM3	calmodulin 3 (phosphorylase kinase, delta)	−0.71
Nucleic acid binding	NM_006450	SPF45	splicing factor (45 kD)	−0.75
	NM_001950.2	E2F4	E2F transcription factor 4, p107/p130-binding	−0.68
	NM_024038.2	MGC2803	hypothetical protein MGC2803	−0.86
	NM_004111.4	FEN1	flap structure-specific endonuclease 1	−0.62
	NM_004875.1	POLR1C	polymerase (RNA) I polypeptide C, 30 kDa	−0.64
	NM_003090.1	SNRPA1	small nuclear ribonucleoprotein polypeptide A′	−0.89
	NM_013400.1	REPIN1	replication initiator 1	−0.66
	NM_003158	STK6	serine/threonine kinase 6	−0.98
Response to external stimulus	NM_003226.1	TFF3	trefoil factor 3 (intestinal)	−0.88
Morphology	NM_006000.1	TUBA1	tubulin, alpha 1 (testis specific)	−0.60
Miscellaneous	NM_021821.2	MRPS35	mitochondrial ribosomal protein S35	−0.65
	NM_006585.1	CCT8	chaperonin containing TCP1, subunit 8 (theta)	−0.70
	NM_031434.2	C7orf21	chromosome 7 open reading frame 21	−0.79
	NM_017566.2	DKFZp434G0522	hypothetical protein DKFZp434G0522	−0.65
	NM_177525.1	MEST	mesoderm specific transcript homolog (mouse)	−0.71
	NM_007019.2	UBE2C	ubiquitin-conjugating enzyme E2C	−0.64

a) Expression level indicates the natural logarithm of the Cy5/Cy3 ratio of the expression value.

Table 4. Primers used for RT-PCR

Gene product	Forward primer	Reverse primer
p21	TGC CAA GCT TCT AAG ATT TC	GGA GAG AGG AAA AGG AGA AC
HIF-1 α-subunit Inhibitor	GCA CTT AAT GGA CTG GAC TC	CCA AGA GCA TCT CAT CTC TC
GATA-3	TAT CAC AAA ATG AAC GGA CA	GTT AAA CGA GCT GTT CTT GG
PLK1	CTC AAT AAA GGC TTG GAG AA	CTT CTT CAT CAA GGA GTT GG
CyclinB2	AGT GTC CTC CCT TTT CAG TC	AGC CAA CTT TTC CAT CTG TA
MADD	GCC TGA AGT AAT CAA ACC TG	GCA GAG TTG GTC TCA ATT TC
P450	GCC TGA TCA CTC CTA CAG AG	ACT TCA GGA TGT TGA AAT GG
CypB	ATT CCA TCG TGT AAT CAA GG	CTC AAA GAA AGA TGT CCC TG
β-Actin	GCT CGT CGT CGA CAA CAA CGG	CAA ACA TGA TCT GGG TCA TCT

this through an alteration of the spindle checkpoint to the chromosomal instability observed in colorectal cancer.[25] The genes involved in cell growth and/or maintenance were also downregulated by TSA. Elevated expression of mammalian polo-like kinase 1 (Plk1) occurs in many different types of cancers, and it is reported that Plk1 depletion dramatically inhibited cell proliferation, decreased viability, and resulted in cell-cycle arrest.[26]

The down-regulated 22 genes by AdoHcy treatment include certain genes involved in certain cytoplasmic and nuclear signaling pathways, such as calcium signaling, cell growth, cell proliferation, immune response, transcriptional initiation, and pentose signaling (Table 2B). Cyclophilin B (CypB) is recognized by tumor-specific cytotoxic T lymphocytes, and CypB-derived peptides have an ability to generate anti-tumor immune responses.[27]

The Genes Commonly Regulated by Both TSA and AdoHcy Although a clear physiological meaning of the close connection between the inhibition of deacetylation and methylation are not known, identifying the genes commonly regulated in both drug treatments in the gastric cancer cell line should provide information for further study. To identify the genes commonly up- and down-regulated in both treatments, we merged all the array data from TSA and AdoHcy treatments, and filtered the genes that present ratio data of more than 80%. A total of 3136 genes were selected from both drug treated sets as a result of filtering. The selected genes designated as a single class in SAM analysis and a *t*-test was performed. Those genes in the range of FDR values of less than 0.15% and average fold change of more than 1.5 were classified with gene ontology. The genes associated with kinase activity and metal ion binding were among the notable up-regulated groups in both drug treatments (Table 3A). Various nucleic acid binding proteins were among the group of gene products commonly downregulated by both drugs (Table 3B). A close relationship between different histone modifications such as acetylation and methylation has recently been suggested. The methylated lysine 4 and acetylated lysine 9/14 of histone H3 were localized to transcriptionally active genes. It has been shown that PRMT1-methylated histone H4 becomes a better substrate for p300 and, conversely, that acetylated histones are poor substrates for methylation by PRMT1.[28] Thus, those genes differentially expressed by both drugs are up- and down-regulated in the hyperacetylation and hypomethylation state of histones. It is postulated that the hyperacetylation of histone is associated with the activation of gene transcription, however, histone methylation is related to both transcriptional activation and repression, depending on the site of methylation. Further examination of the gene expression profile when both drugs are treated at the same time, and comparison of them with our data may provide valuable information.

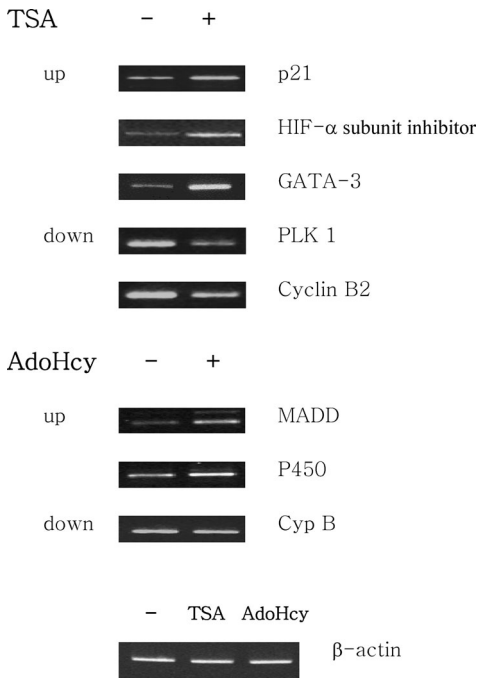

Fig. 2. RT-PCR Analysis of Gene Expression Data from Up-Regulated and Down-Regulated by TSA and AdoHcy Treatments

The β-actin amplification was carried out as an internal control.

Validation of Microarray Data Using RT-PCR To verify the reliability of the expression changes detected by the profiling analysis using the cDNA microarray, RT-PCR analysis with the same RNA samples that had been used for the microarray was performed for the elective number of genes. The expression profiles of tested genes by RT-PCR coincide with the expression profiles in microarray analysis (Fig. 2). We analyzed p21, HIF-1 α subunit inhibitor, GATA-3 for up-regulated genes with TSA treatment and PLK1, cyclin B2 for down-regulated genes with TSA treatment. Also, we tested MADD, cytochrome P450 and CypB for up-regulation and down regulation with AdoHcy treatment, respectively.

Determination of Apoptosis Although HDAC inhibitor TSA has been known to cause apoptosis in many tumors, the

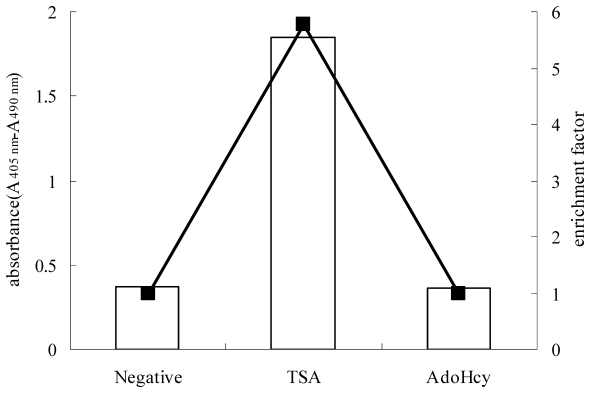

Fig. 3. Enrichment of Nucleosomes in the Cytoplasm of Cells Treated with TSA and AdoHcy

DNA fragmentation is presented as a nucleosome enrichment factor compared to the respective untreated control. The bar graphs indicate absorbance, and linear graphs indicate enrichment factors. Results are mean±S.D. from $n=2$, done in duplicate.

role of AdoHcy in cell death signaling has been poorly understood. To investigate the apoptotic status of cells when microarrays were performed, cells were treated under the same conditions as microarray analysis, and cell death was quantified. The assay revealed that while TSA-treated cells showed significantly increased nucleosome formation, AdoHcy-treated cells remained at the same cell death ratio as untreated cells (Fig. 3). The apoptotic activity of TSA is known to be confined to transformed cells. Treatment of normal human cells with HDAC inhibitors caused no apoptosis. This indicates that apoptosis in the gastric cancer cell line by TSA in this study is very specific, through the mechanism involved in chromatin structure. Also, TSA induces apoptosis only in certain types of cancers, and cell death induction in other types of cells might be through the other pathways involving other genes. This study also indicates AdoHcy roles other than involvement in apoptosis in the gastric cancer cell line SNU-16.

In conclusion, our data show gene expression profiling based on a cDNA microarray analysis of gastric cancer cell line treated with TSA and AdoHcy. Comparison of the differentially and commonly expressed genes between two different inhibitors of post-translational modifications, including histone modifications, should provide valuable information for further research on understanding the molecular mechanism of gene expression in gastric carcinogenesis. Further research on these identified genes will provide potential new insights into cancer biology by correlating two different post-translational modification conditions in gastric cancer.

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