Recovery of Genes Epigenetically Altered by the Histone Deacetylase Inhibitor Scriptaid and Demethylating Agent 5-Azacytidine in Human Leukemia Cells

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Abstract

Histone deacetylation and demethylation are epigenetic mechanisms implicated in cancer. Studies regarding the role of modulation of gene expression utilizing the histone deacetylase inhibitor scriptaid and the demethylating agent 5-azacytidine in HL-60 leukemia cells have been limited. We studied the possibility of recovering epigenetically silenced genes by scriptaid and 5-azacytidine in human leukemia cells by DNA microarray analysis. The first group was leukemia cells that were cultured with 5-azacytidine. The second group was cultured with scriptaid. The other group was cultured with both agents. Two hundred seventy newly developed genes were expressed after the combination of 5-azacytidine and scriptaid. Twenty-nine genes were unchanged after the combination treatment of 5-azacytidine and scriptaid. Among the 270 genes, 13 genes were differed significantly from the control. *HPGD*, *CPA3*, *CEACAM6*, *LOC653907*, *ETS1*, *RAB37*, *PMP22*, *FST*, *FOXC1*, and *CCL2* were up-regulated, and *IGLL3*, *IGLL1*, and *ASS1* were down-regulated. Eleven genes associated with oncogenesis were found among the differentially expressed genes: *ETS1*, *ASCL2*, *BTG2*, *BTG1*, *SLAMF6*, *CDKN2D*, *RRAS*, *RET*, *GIPC1*, *MAGEB*, and *RGL4*. We report the results of our leukemia cell microarray profiles after epigenetic combination therapy with the hope that they are the starting point of selectively targeted epigenetic therapy.

Keywords: deacetylation, demethylation, epigenetic, leukemia, microarray

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Introduction

Chromatin remodeling is important in gene expression, DNA repair, and replication (Bishton *et al.*, 2007). Methylation of promotor regions of tumor suppressor genes and histone deacetylation lead to gene silencing (Bishton *et al.*, 2007). Epigenetic modification with histone deacetylase inhibitors and hypomethylating agents allow upregulation of proapoptotic genes and downregulation of antiapoptotic genes, and show significant single-agent anticancer activity (Bishton *et al.*, 2007; Dalgard *et al.*, 2008). Combinations of these drugs are being employed in clinical trials to target multiple biological pathways (Bishton *et al.*, 2007). Preclinical studies of combinations of these agents are ongoing and demonstrate synergy in many hematological cancers (Bishton *et al.*, 2007). But, the exact target is still ambiguous and can not be controlled precisely. We report the results of our leukemia cell microarray profiles after epigenetic combination therapy.

There are several reasons why both demethylating agents and histone deacetylase inhibitors (HDACis) are excellent candidates for examination as part of combination strategies (Bishton *et al.*, 2007). First, these agents have considerable single-agent activity in hematological malignancies; this activity is probably related to the modification of epigenetic changes, leading to re-expression of aberrantly silenced tumor suppressor genes (Bishton *et al.*, 2007). However, the relatively low response of single agents in myelodysplastic syndromes and acute myeloid leukemia demonstrates that there are numerous cellular 'escape mechanisms' that allow the cell to avoid cell death (Bishton *et al.*, 2007). Combination approaches with demethylating agents and HDACis that are targeted at the epigenome may overcome some of this resistance. Second, a multitarget hit strategy seems to benefit cancer therapy. As we dissect critical cell cycle, proliferation, apoptosis, angiogenesis, and signaling pathways, we can develop molecules to target them (Bishton *et al.*, 2007). It is clear that in addition to the effect on histone acetylation, non-histone targets are critical for the effects of these drugs. Such targets for hyperacetylation include p53, α-tubulin, HIF-1α, and HSP90 (Bali *et al.*, 2005; Huang *et al.*, 2002; Kawaguchi *et al.*, 2003; Qian *et al.*, 2006). Finally, the good toxicity profile of these agents make them suitable for combina-

Table 1. Drug combination density options to determine the effect of the combination of 5-azacytidine and scriptaid

Solution (2 ml)	Control	A 2.5 μl+S 2.5 μl	A 5 μl+S 5 μl	A 10 μl+S 10 μl	A 20 μl+S 20 μl
1st set (O.D.)	2.02	0.7	0.3	0.24	0.25
2nd set (O.D.)	2.27	0.51	0.38	0.27	0.27
3rd set (O.D.)	2.56	0.52	0.4	0.37	0.24

O.D. stands for optical density. A stands for 5-azacytidine, and S stands for scriptaid.
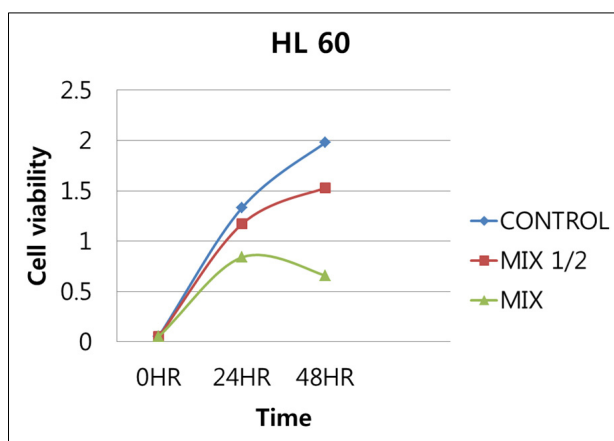

Fig. 1. HL-60 cell viability curve after treatment with 5-azacytidine and scriptaid. Mix 1/2 group stands for the half-dose of each drug combination compared with the MIX group.
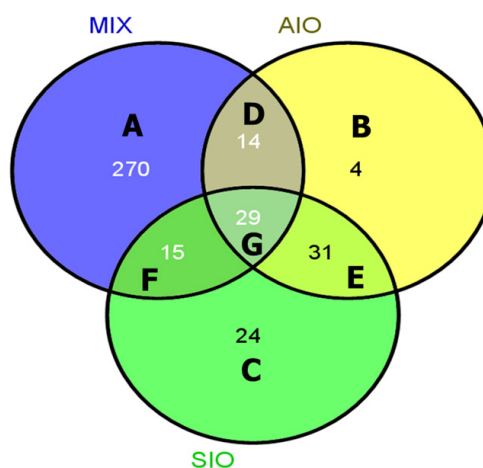

Fig. 2. Expressed gene numbers in leukemia cells after treatment with 5-azacytidine, scriptaid, and the combination regimen (AIO: 5-azacytidine; SIO: Scriptaid; MIX: treatment with both 5-azacytidine and scriptaid).

tion therapy (Rasheed *et al.*, 2007).

Methods

Cell lines & Cell culture

The HL-60 leukemic cell line was obtained from Korean Cell Line Bank (KCLB). Cell lines were cultured at 37°C (5% CO$_2$) in RPMI 1640 medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (all purchased from Sigma-Aldrich Co. Ltd. Poole, UK).

Determination of effects of drug combination

5-azacytidine (12.25 μM) and 0.83 μM scriptaid were used. A 2-mL solution of control and 5-azacytidine 2.5 μL, scriptaid 2.5 μL combination regimen were compared (Table 1). Cell viability curves were drawn from the combination-treated cells (Fig. 1).

WST-1 assay

Cell number and viability were further assessed by using the WST-1 reagent (Roche, Indianapols, USA). This col-

orimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 into formazan by mitochondrial dehydrogenase in live cells. Cells under different treatments were plated at 1×10^4 cells/0.32 cm^2 in a 96-well plate and were maintained for 48 hr at 37°C in 8.5% CO$_2$. Absorbance readings were normalized against control wells with medium alone. All experiments were performed independently at least 3 times in triplicate per experimental point.

RNA extraction

Total RNA was isolated from cell lines using Nucleospin RNA II (Macherey-Nagel, Germany). RNA quality was assessed using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA) spectrophotometer. Extracted RNA was stored at -70°C.

Total RNA yield was quantified by densitometric assessment of both ribosomal bands using the FlourChem Digital Imaging System (Alpha Inotek, Inc., San Leandro, CA, USA).

Microarray analysis

The microarray hybridization was performed using the Illumina Gene Expression 48K Chip (Illumina). Data visualization, clustering, and differential analysis were carried out with BeadStudio software (Illumina) and the GeneSpring GX program, 7.3 (Silicon Genetics).
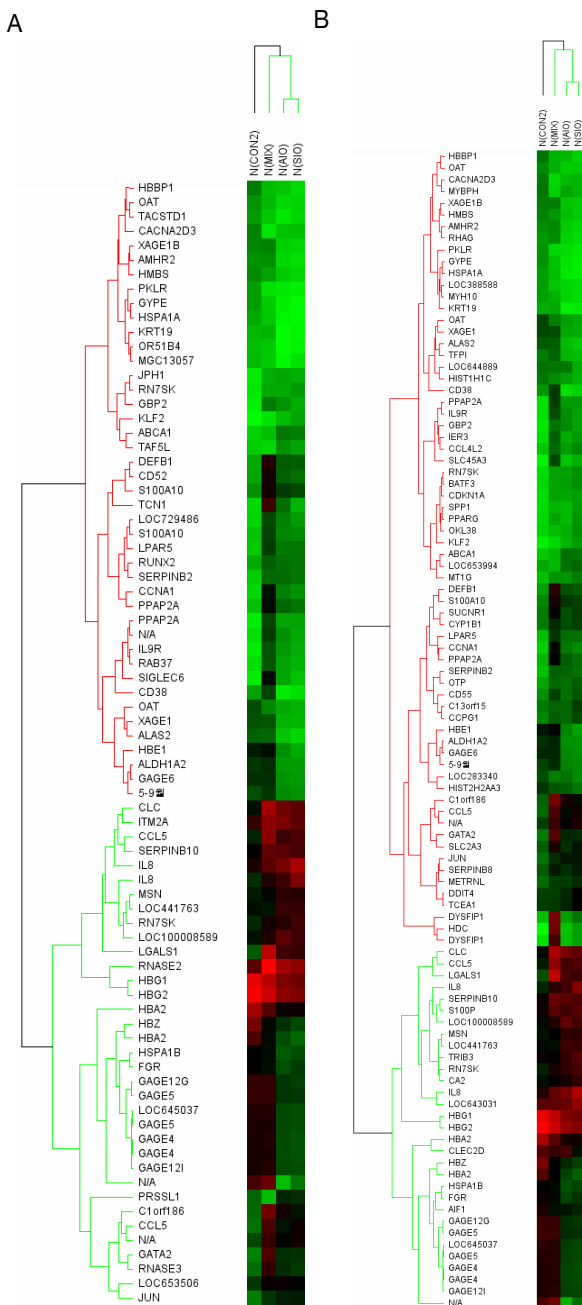

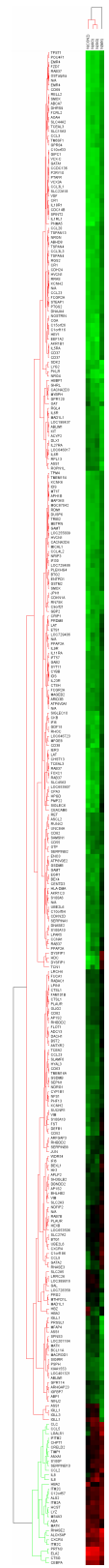**Fig. 3.** Gene expression profile of leukemia cells after treatment. (A) 5-Azacytidine, (B) Scriptaid.

Fig. 4. Gene expression profile of leukemia cells after treatment with combination regimen of 5-azacytidine and scriptaid.

Results

Determination of effects of drug combination

The 5-azacytidine and scriptaid combination regimen-treated leukemia cells were decreased after 48 hours of incubation. The demethylating agent and HDACi combination regimen can be an option to decrease leukemia cells (Fig. 1).

Differentially expressed genes in leukemia cells treated with 5-azacytidine, scriptaid, or the combination regimen

There were 78 expressed genes after treatment with 5-azacytidine (Fig. 2, 3). Ninety-nine genes were expressed after treatment with scriptaid (Fig. 2, 3). Sixty genes overlapped between the 5-azacytidine treatment and scriptaid treatment (Fig. 2).

After treatment with both 5-azacytidine and scriptaid, which is a combination of a demethylating agent and an HDACi, there was a remarkable gene expression profile change (Fig. 4, Table 2). Two hundred seventy newly developed genes were expressed (Fig. 2, Table 2). The gene ontology biological process profile shows that of these 270 genes, 94 genes were associated with signal transduction (34.81%), 68 genes were associated with immunity and defense (25.19%), and 35 genes were associated with developmental processes (12.96%) (Fig. 4, Table 2). The gene ontology molecular function profile shows that 32 genes were associated with receptors (11.85%), 23 genes were associated with signaling molecules (8.52%), and 19 genes were associated with select regulatory molecules (7.04%) (Fig. 5, Table 2).

We selected 13 genes that showed higher fold-changes after treatment with combination therapy with 5-azacytudune and scriptaid. *HPGD*, *CPA3*, *CEACAM6*, *LOC653907*, *ETS1*, *RAB37*, *PMP22*, *FST*, *FOXC1*, and *CCL2* were up-regulated, and *IGLL3*, *IGLL1*, and *ASS1* were down-regulated (Table 3).

Differentially expressed genes associated with oncogenesis in leukemia cells treated with the 5-azacytidine and scriptaid combination regimen

Of the 270 genes, 11 genes were involved in oncogenesis: *ETS1*, *ASCL2*, *BTG2*, *BTG1*, *SLAMF6*, *CDKN2D*, *RRAS*, *RET*, *GIPC1*, *MAGEB*, and *RGL4* (Fig. 2, Table 4).

Twenty-nine genes remained unchanged after combination treatment with 5-azacytidine and scriptaid (Fig. 2, Table 5). Two genes were involved in oncogenesis: *CCNA1* and *JUN* (Fig. 2, Table 5).

Table 2. Functional annotation of differentially expressed genes in leukemia cells after treatment with combination regimen of 5-azacytidine and scriptaid

Category	Term	Count (%)
GO BP	Signal transduction	94 (34.81)
	Immunity and defense	68 (25.19)
	Developmental processes	35 (12.96)
	Protein metabolism and modification	25 (9.26)
	Nucleoside, nucleotide and nucleic acid metabolism	21 (7.78)
	Cell proliferation and differentiation	20 (7.41)
	Cell cycle	16 (5.93)
	Transport	14 (5.19)
	Cell adhesion	12 (4.44)
	Cell structure and motility	12 (4.44)
	Lipid, fatty acid and steroid metabolism	12 (4.44)
	Oncogenesis	11 (4.07)
	Other metabolism	9 (3.33)
	Carbohydrate metabolism	8 (2.96)
	Intracellular protein traffic	8 (2.96)
	Neuronal activities	7 (2.59)
	Apoptosis	7 (2.59)
	Amino acid metabolism	6 (2.22)
	Sulfur metabolism	4 (1.48)
	Sensory perception	3 (1.11)
	Homeostasis	2 (0.74)
	Miscellaneous	2 (0.74)
	Muscle contraction	2 (0.74)
	Nitrogen metabolism	2 (0.74)
	Coenzyme and prosthetic group metabolism	1 (0.37)
	Phosphate metabolism	1 (0.37)
	Protein targeting and localization	1 (0.37)
GO MF	Receptor	32 (11.85)
	Signaling molecule	23 (8.52)
	Select regulatory molecule	19 (7.04)
	Defense/immunity protein	16 (5.93)
	Transcription factor	16 (5.93)
	Oxidoreductase	14 (5.19)
	Nucleic acid binding	13 (4.81)
	Miscellaneous function	12 (4.44)
	Cell adhesion molecule	11 (4.07)
	Hydrolase	10 (3.70)
	Protease	10 (3.70)
	Transporter	9 (3.33)
	Kinase	7 (2.59)
	Phosphatase	6 (2.22)
	Transferase	6 (2.22)
	Ligase	5 (1.85)
	Cytoskeletal protein	4 (1.48)
	Membrane traffic protein	4 (1.48)
	Extracellular matrix	3 (1.11)
	Lyase	3 (1.11)
	Synthase and synthetase	2 (0.74)
	Chaperone	1 (0.37)
	Ion channel	1 (0.37)
	Select calcium binding protein	1 (0.37)

BP: biological process, GO: gene ontology, MF: molecular function. Count indicates the number of genes assigned to the gene ontology term.

Discussion

Demethylating agents, such as 5-azacytidine and decitabine, are actively implicated in clinical practice to improve survival and quality of life in myelodysplastic syndrome. Their potential action on acute leukemia is also being studied. HDACis, such as scriptaid and valproic acid, are also useful in decreasing leukemia cells (Chavez-Blanco *et al.*, 2006). But, neither of these single agent makes clinicians satisfied as a cure for acute leukemia. There are a few studies that have combined these two epigenetic therapies to reveal silenced tumor suppressor genes. The dual epigenetic therapy may induce positive or negative genetic alterations in leukemia cells, and the gene expression profile may change a lot after combining the two epigenetic therapies--a demethylating agent and an HDACi. There are little data on the gene expression profiles of dual epigenetic therapy so far.

It is notable that over two times as many genes were newly expressed after treatment with both the demethylating agent and HDACi. The newly expressed genes could work positively or negatively on leukemia cells. So, before applying dual epigenetic therapy to the clinical setting, target gene information may help clinicians to understand the mechanisms of leukemogenesis and epigenetic therapy.

Our results show that the major portion of newly expressed genes after combination treatment with 5-aza-

cytidine and scriptaid are associated with signal transduction and immunity. The associated signal trans-
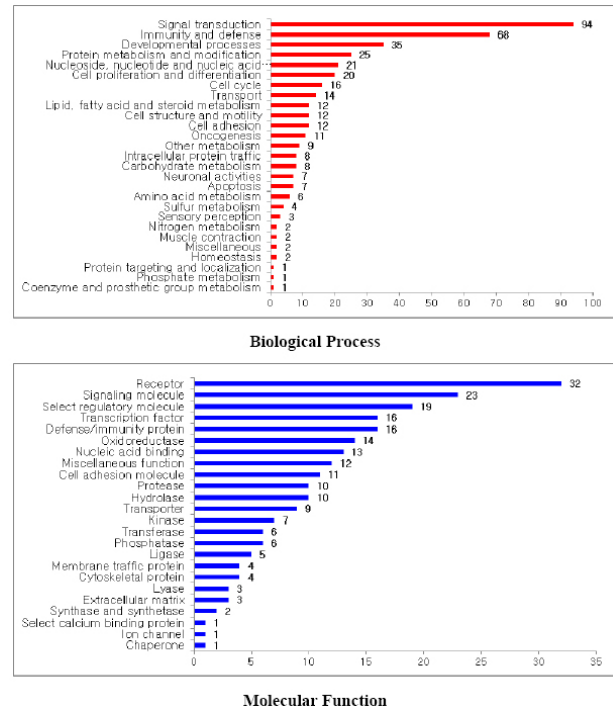

Fig. 5. Gene ontology of gene expression profile of leukemia cells after treatment with combination regimen of 5-azacytidine and scriptaid.

Table 3. Differentially expressed genes in leukemia cells treated with combination regimen with 5-azacytidine and scriptaid

Gene	Reporter ID	GO BP	GO MF	Fold
HPGD	NM_000860.3	Oxidoreductase	Lipid, fatty acid and steroid metabolism	9.24
CPA3	NM_001870.1	Protease	Protein metabolism and modification	8.55
CEACAM6	NM_002483.3	Cell adhesion molecule	Signal transduction	7.44
LOC653907	XM_936496.2	Receptor	Immunity and defense	7.18
ETS1	NM_005238.2	Signaling molecule	Nucleoside, nucleotide and nucleic acid metabolism; Oncogenesis	6.76
RAB37	NM_175738.3	Select regulatory molecule	Signal transduction	6.70
PMP22	NM_153321.1	Cytoskeletal protein	Neuronal activities	6.63
FST	NM_013409.1	Select regulatory molecule	Homeostasis	5.49
FOXC1	NM_001453.1	Transcription factor	Carbohydrate metabolism; Nucleoside, nucleotide and nucleic acid metabolism; Signal transduction; Sensory perception; Developmental processes; Cell cycle; Cell proliferation and differentiation; Cell structure and motility	5.29
CCL2	NM_002982.3	Signaling molecule	Signal transduction; Immunity and defense	5.17
IGLL3	NM_001013618.1	Defense/immunity protein	Immunity and defense	-7.10
IGLL1	NM_020070.2	Defense/immunity protein	Immunity and defense	-8.76
ASS1	NM_000050.4	Ligase	Amino acid metabolism; Nitrogen metabolism	-9.02

BP: biological process, GO: gene ontology, MF: molecular function. The fold-change is the ratio of average expression values of combination treatment specimens to those of untreated (control) specimens. A positive and negative score indicates up-regulation and down-regulation in the combination regimen-treated specimens, respectively.

Table 4. Newly expressed genes associated with onco-genesis in leukemia cells after treatment with the combination regimen of 5-azacytidine and scriptaid

Gene	Reporter ID	Fold change
ETS1	NM_005238.2	6.76
ASCL2	NM_005170.2	3.18
BTG2	NM_006763.2	3.17
BTG1	NM_001731.1	2.91
SLAMF6	NM_052931.3	2.74
CDKN2D	NM_079421.2	2.41
RRAS	NM_006270.3	2.39
RET	NM_020630.4	2.37
GIPC1	NM_202468.1	2.29
MAGEB2	NM_002364.3	2.22
RGL4	NM_153615.1	−2.68

The fold-change is the ratio of average expression values of combination treatment specimens to those of untreated (control) specimens. A positive and negative score indicates up-regulation and down-regulation in the combination regimen-treated specimens, respectively.

Table 5. Unchanged genes associated with oncogenesis in leukemia cells after treatment with combination regimen of 5-azacytidine and scriptaid

Gene	Reporter ID	Fold change
CCNA1	NM_003914.2	10.37
JUN	NM_002228.3	2.33

The fold-change is the ratio of average expression values of combination treatment specimens to those of untreated (control) specimens. A positive and negative score indicates up-regulation and down-regulation in the combination regimen-treated specimens, respectively.

duction pathways may be the key to leukemogenesis and a cure for leukemia. But, due to the limitations of cell line data, our results can not generate an answer yet. Further clinical confirmation is needed.

HPGD, hydroxyprostaglandin dehydrogenase 15, encodes a member of the short-chain nonmetalloenzyme alcohol dehydrogenase protein family. The encoded enzyme is responsible for the metabolism of prostaglandins, which function in a variety of physiological and cellular processes, such as inflammation. Mutations in this gene result in primary autosomal recessive hypertrophic osteoarthropathy and cranioosteoarthropathy. A recent study reported the association of a risk of lung cancer with an *HPGD* SNP (Wang *et al.*, 2010). It is inversely correlated with VEGF expression in colorectal cancer (Lim *et al.*, 2010). *HPGD* is down-regulated in gastric cancer (Thiel *et al.*, 2009). Reduction of *HPGD* expression is an independent predictor of poor survival, associated with enhanced cell proliferation in gastric adenocarcinoma (Tatsuwaki *et al.*, 2010). Down- regulation of *HPGD* contributes to PGE_2 accumulation in lung, colon, and pancreatic cancers (Pham *et al.*, 2010). But, *HPGD* protein level is higher in malignant ovarian tissue samples compared to normal tissue, which does not correlate with the former report (Thill *et al.*, 2010). *HPGD* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

CPA3, carboxypeptidase A3, encodes mast cell carboxypeptidase A (Mc-cpa), which is a highly conserved secretory granule protease. Mc-cpa plays a crucial role in the generation of phenotypically mature mast cells

(Feyerabend *et al.*, 2005). *CPA3* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

Overexpression of *CEACAM6*, carcinoembryonic antigen-related cell adhesion molecule 6, independently predicted poor overall survival in colorectal cancer (Jantscheff *et al.*, 2003). High expression of *CEACAM6* mRNA was reported in acute lymphoblastic leukemias (Lasa *et al.*, 2008). *CEACAM6* is associated with neutrophil adhesion to the endothelium and angiogenesis (Skubitz and Skubitz 2008). *CEACAM6* is a major target gene for Smad3-mediated TGF-beta signaling (Han *et al.*, 2007). There is a report of CEACAM6 overexpression in borderline and invasive ovarian neoplasms (Litkouhi *et al.*, 2008). *CEACAM6* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

LOC653907 is similar to complement component (3b/4b) receptor 1 isoform F precursor. It is associated with immunity and defense. There are some reports that it is associated with Alzheimer disease or dementia (Bossers *et al.*, 2010). *LOC653907* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

ETS1, erythroblastosis virus E26 oncogene homolog 1, regulates numerous genes and is involved in stem cell development, cell senescence and death, and tumorigenesis (Russell and Garrett-Sinha, 2010). *ETS1* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

RAB37 is a member of the RAS oncogene family. Rab proteins are low-molecular-mass GTPases that are critical regulators of vesicle trafficking. Frequent down-regulation of *RAB37* in lung cancer has been reported (Wu *et al.*, 2009). *RAB37* with renal cell cancer association has also been reported (Dobashi *et al.*, 2009). *RAB37* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

PMP22, peripheral myelin protein 22, mediates Schwann cell growth and peripheral myelin compaction. Interleu-

kin-6 upregulates the expression of *PMP22* in cultured rat Schwann cells via a JAK2-dependent pathway (Ito *et al.*, 2010). *PMP22* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

FST, follistatin, is a single-chain gonadal protein that specifically inhibits follicle-stimulating hormone release. Overexpression of follistatin in human myoblasts increases their proliferation and differentiation and improves graft success in SCID mice (Benabdallah *et al.*, 2009). *FST* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

FOXC1, forkhead box C1, belongs to the forkhead family of transcription factors, which are characterized by a distinct DNA-binding forkhead domain. The specific function of this gene has not yet been determined; however, it has been shown to play a role in the regulation of embryonic and ocular development. Mutations in this gene cause various glaucoma phenotypes, including primary congenital glaucoma, autosomal dominant iridogoniodysgenesis anomalies, and Axenfeld-Rieger anomalies. There is frequent aberrant DNA methylation of FOXC1 in breast cancer (Muggerud *et al.*, 2010). *FOXC1* is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

CCL2, chemokine ligand 2, encodes a monocyte chemoattractant protein. It is up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

IGLL3, immunoglobulin lambda-like polypeptide 3, and *IGLL1*, immunoglobulin lambda-like polypeptide 1, are down-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid. *ASS1*, argininosuccinate synthetase 1, is also down-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

Among the 270 genes, 11 genes are involved with oncogenesis. They could be oncogenes or tumor suppressor genes. So, newly expressed tumor suppressor genes, such as *CDKN2D*, could function to suppress hematological malignancies. But, newly developed oncogenes could be the cause of secondary malignancies or progression of existing hematological malignancies.

ASCL2, achaete-scute complex homolog 2, controls intestinal stem cell fate (van der Flier *et al.*, 2009). It is a target of *Wnt* signaling and is upregulated in intestinal neoplasia (Jubb *et al.*, 2006).

BTG2, B-cell translocation gene 2, is a tumor suppressor gene of medulloblastoma (Farioli-Vecchioli *et al.*, 2007). *BTG2* plays a pivotal role in maintaining the hematopoietic stem cell compartment and hematopoiesis (Kim *et al.*, 2008). *BTG1* is associated with angiogenesis (Iwai *et al.*, 2004).

SLAMF6 is an oncogene associated with B cell adhe-

sion (Cannons *et al.*, 2010). *CDKN2D*, cyclin-dependent kinase inhibitor 2D (p19), and RRAS, Harvey rat sarcoma oncogene, subgroup R, are also up-regulated in leukemia cells after combination treatment with 5-azacytidine and scriptaid.

Genetic alterations in the 29 genes that were unchanged after treatment with both the demethylating agent and HDACi might give us solutions to overcome hematological malignancies. There are two genes involved in oncogenesis, *CCNA1* and *JUN* (Fig. 1, Table 4). These two genes could be the target genes to overcome with other chemotherapeutic agents, monoclonal antibodies, or small molecules which cannot be controlled with epigenetic therapy.

There is some evidence that combination strategies with multiple epigenetic agents, such as demethylating agents and HDACis, show synergistic effects and benefits in hematological malignancies (Bishton *et al.*, 2007; Cameron *et al.*, 1999; Chavez-Blanco *et al.*, 2006; Desmond *et al.*, 2007; Galm *et al.*, 2006). But, the lack of long-term follow-up of clinical data and the exact genetic information after treatment with multiple epigenetic therapy makes clinicians hesitate in using epigenetic combination therapy. Demethylating agents, such as 5-azacytidine and decitabine, are nonselective hypomethylating agents that can not selectively target specific chromosomal loci. But, there are synergistic gene expression changes after multiple epigenetic therapy, and information about the target genes could be the weapons to conquer hematological malignancies. There are no previous reports of microarray genetic profiles after treatment with both the demethylating agent 5-azacytidine and the HDACi scriptaid. We report our results on leukemia cell microarray profiles after epigenetic combination therapy with the hope that they are the starting point of selectively targeted epigenetic therapy.

Nonetheless, combination trials will need to be designed carefully, as it is possible that unexpected toxicities may be unearthed. Transient thrombocytopenia is a common dose-dependent toxic effect of HDACis, which may limit our ability to combine these drugs with chemotherapy (Bishton *et al.*, 2007). Large randomized trials will be required to establish that combination approaches are advantageous.

To further our understanding of the targets of these epigenetic agents, careful preclinical studies of mechanisms and pharmacodynamics analysis, incorporated within translational studies on clinical trials, are essential. As our knowledge of the epigenetic control of gene expression expands, novel targets for therapeutic intervention will be identified, and new classes of selectively targeted agents will be developed.

Acknowledgments

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