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Suberoylanilide hydroxamic acid (SAHA) changes microRNA expression profiles in A549 human non-small cell lung cancer cells

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Abstract. Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase inhibitor (HDACI) with antitumor effects that is being explored as a therapeutic drug. However, it has been reported that non-small cell lung cancer (NSCLC) is resistant to HDACIs. MicroRNAs (miRNAs) are a key class of small, non-coding RNA molecules that modulate post-transcriptional regulation of gene expression in multi-cellular organisms. miRNA expression patterns are involved in deregulation of gene expression in human lung cancer. Here we identified miRNA expression profile changes in response to SAHA treatment in the human lung carcinoma cell line A549. We also examined potential mRNA targets of SAHA-responsive miRNAs by using a target prediction program. Using microarray analysis, we found 64 miRNAs with >2-fold expression changes in SAHA-treated A549 cells. Among them, two unique miRNAs were altered in 2.5 μM SAHA-treated cells, 31 unique miRNAs were altered in 5.0 μM SAHA-treated cells and 31 miRNAs were altered with both doses. These miRNAs are predicted to have several target genes related to angiogenesis, apoptosis, chromatin modification, cell proliferation and differentiation. In conclusion, we have identified a unique set of miRNAs and their expression profiles that are influenced significantly by SAHA in the A549 NSCLC cell line model, which might provide useful information for understanding the anticancer mechanism of SAHA.

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Introduction

Several studies have shown that histone deacetylases (HDACs) regulate many cellular functions. Erroneous phenotypic changes such as developmental disorders and cancer can be caused by aberrant histone acetylation (1). The regulation of histone deacetylation by HDACs modulates transcriptional regulation (2). In addition, certain types of human cancers are associated with abnormal deacetylation (3). Histone deacetylase inhibitors (HDACIs) are emerging as the target of several anticancer agents, which affect tumor cell proliferation, differentiation and induction of cell death in diverse cancer types (4-12). Suberoylanilide hydroxamic acid (SAHA) is an HDACI, and has been studied in several phase I/II clinical trials as a therapeutic cancer drug (13,14). However, the antitumor mechanism of HDACIs is not completely understood. Many researchers have investigated the effects of HDACI treatment in cancer patients and have obtained variable results. Non-small cell lung cancer (NSCLC) has been shown to be resistant to HDACIs such as trichostatin A and SAHA (2,15). For this reason, NSCLC has been treated in combination with other antitumor agents (16).

MicroRNAs (miRNA) are small non-coding RNA molecules that modulate the post-transcriptional regulation of gene expression in multi-cellular organisms by complementary interaction with the 3'-untranslated regions (3'-UTR) of target mRNA (17-19). miRNA interaction influences protein expression by promoting the degradation or suppressing mRNA translation (20,21). Several studies have shown that only a few miRNA expression patterns have been involved in deregulation of gene expression in human lung cancer. The role of miRNAs are not completely understood, however several studies have shown a correlation between miRNAs and human lung cancer (22-24).

We hypothesized that SAHA treatment of lung cancer cells could affect miRNA expression, and it may clarify the antitumor effects of HDACIs. We investigated whether SAHA treatment influences miRNA expression profiles in A549 cells using miRNA microarray. We discovered changes in miRNA expression and identified SAHA-responsive miRNAs in A549 cells. In addition, the target genes of the

candidate miRNAs may provide valuable information for cancer research.

Materials and methods

Cell culture. The tumorigenic NSCLC cell line A549 was cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), and antibiotics at 37˚C in a humidified chamber supplemented with 5% CO_2. One day before SAHA treatment, $4x10^5$ cells were seeded into 60-mm culture dishes.

SAHA treatment. SAHA (Alexis Biochemicals, MA, Switzerland) was dissolved in DMSO (Sigma, MO, USA) and added to A549 cultures at different concentrations (2.5 and 5.0 μM). DMSO was added to culture media as a control.

RNA preparation. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, CA, USA) 24 h after SAHA treatment. For the microarray studies, the quality and concentration of the RNA samples was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and an Ultrospec 3300 Pro UV/Visible Spectrophotometer (Amersham Biosciences, NJ, USA). The recommended RNA quality parameters for the microarray analysis are an OD 260/280 ratio in the range of 1.8-2.0, an OD 260/230 ratio greater than 1.8, an 18s/28s rRNA ratio in the range of 1.8-2.1, and an RNA integrity number (RIN) greater than 8.0.

Microarray analysis of miRNA profiles. The human miRNA microarray version 2 kit (Agilent Technologies) containing probes for 723 human and 76 viral miRNAs was adapted to analyze the expression profiles of miRNAs. Before hybridizing, 100 ng of total RNA including miRNAs was dephosphorylated with calf intestine alkaline phosphatase (CIP), and denatured by adding DMSO and heating. The dephosphorylated RNA was labeled with pCp-Cy3 by T4 RNA ligase, and the labeled RNA was purified with a Micro Bio-Spin P-6 column (Bio-Rad Laboratories, USA). The purified RNA was denatured and hybridized to the probes on the microarray at 55˚C, 20 rpm in the Agilent Microarray Hybridization Chamber (Agilent Technologies) for 20 h. The microarray slide was washed and scanned with the Agilent scanner to obtain the microarray image. The numerical data for the miRNA profiles were extracted from the image with the Feature Extraction program (Agilent Technologies). These data were analyzed with GeneSpring GX software version 7.3 (Agilent Technologies).

Classification of miRNAs. Among the total miRNAs probed on the microarray, 723 human miRNAs were selected for further analysis. The miRNAs whose flags were present in at least one sample were filtered and applied to the fold-change analysis. The fold-change analysis was conducted by a factor of 2-fold between 3 groups: A549 control and 2.5 μM and 5.0 μM of SAHA-treated A549.

Target prediction of miRNAs. Candidate miRNAs that changed more than 2-fold following SAHA treatment were chosen for target prediction and analyzed with miRBase Targets Version 5 on the Enright Lab (Wellcome Trust Sanger Institute) website (http://microrna.sanger.ac.uk/targets/v5/). Human genes with experimentally verified functions related to angiogenesis, apoptosis, chromatin modification, cell proliferation and differentiation were selected from the Gene Ontology website (http://www. geneontology.org/). Finally, the genes predicted as targets of the candidate miRNAs and those selected based on gene ontology were aligned by their gene names. Genes appearing in both lists were chosen and listed.

Results and Discussion

To determine whether SAHA treatment of A549 cells (human type II-like epithelial lung cells) affects changes in miRNA expression levels, we analyzed miRNA profiles using the Agilent human miRNA microarray version 2 kit. A total of 723 human miRNAs, excluding 76 human viral miRNAs, were selected to analyze miRNA profiles. Those human miRNAs were continually filtered with flags as present to obtain more defined data using Agilent Genespring software. We selected total 227 human miRNAs as a result of flag-filtration, and these expression profiles are shown in Fig. 1, sorted by SAHA concentration. The color closest to green reflects low expression levels, whereas red color indicates higher expression levels. Although the majority of miRNAs do not show expression changes, SAHA treatment influences certain miRNA expression levels.

To further confirm that miRNA expression patterns are affected by the SAHA dose, we analyzed 227 flagged miRNAs and removed those that did not demonstrate a 2-fold change in expression levels by comparing the control, 2.5 μM and 5.0 μM treated cells. A total of 64 miRNAs were selected for further study (Table I) and their expression profiles are shown in Fig. 2A. Two miRNAs were altered in 2.5 μM treated cells and 31 miRNAs showing considerable expression changes were found in 5.0 μM treated cells. Surprisingly, 31 miRNAs showing more than 2-fold expression changes in 2.5 μM treated cells were also identified in 5.0 μM treated cells (Fig. 2B). In addition, the number of up- or down-regulated miRNAs was derived from the expression patterns in each treated group. We found 24 up-regulated and 9 down-regulated miRNAs in 2.5 μM treated cells. We also found 48 up-regulated and 12 down-regulated miRNAs in 5.0 μM treated cells (Fig. 2C). Although not all miRNAs show similar regulation patterns in both treatments, we identified many miRNAs with 2-fold expression changes that were regulated by SAHA.

Inhibition or activation of angiogenic gene expression by HDACIs result in anti-angiogenic effects and modulate cell proliferation in a diverse number of cancer cells (25-27). HDACIs induce apoptosis and inhibit tumor cell growth by targeting genes related to chromatin modification (28,29). Thus, we performed target prediction of miRNAs with those genes. A total of 17 miRNAs with expression levels regulated by SAHA treatment in Table I were selected to identify potential target genes related to angiogenesis, apoptosis, chromatin modification and cell proliferation and differentiation. Nearly 1,000 latent targets of each miRNA (excluding miR-1227, which is not available in the program) were predicted using miRBase Target Version 5. We investigated related targets from the Gene Ontology website that were

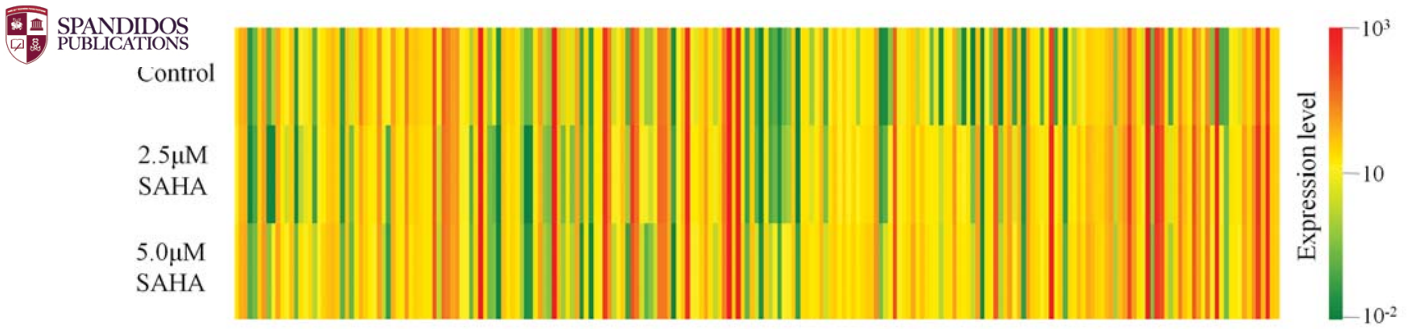

Figure 1. miRNA expression profiles using microarray analysis. After flag-filtration, a total of 227 human miRNA expression levels are represented by color from 10^{-2} (green) to 10^{3} (red). The data were separated into three groups: control, 2.5 μM SAHA and 5.0 μM SAHA.

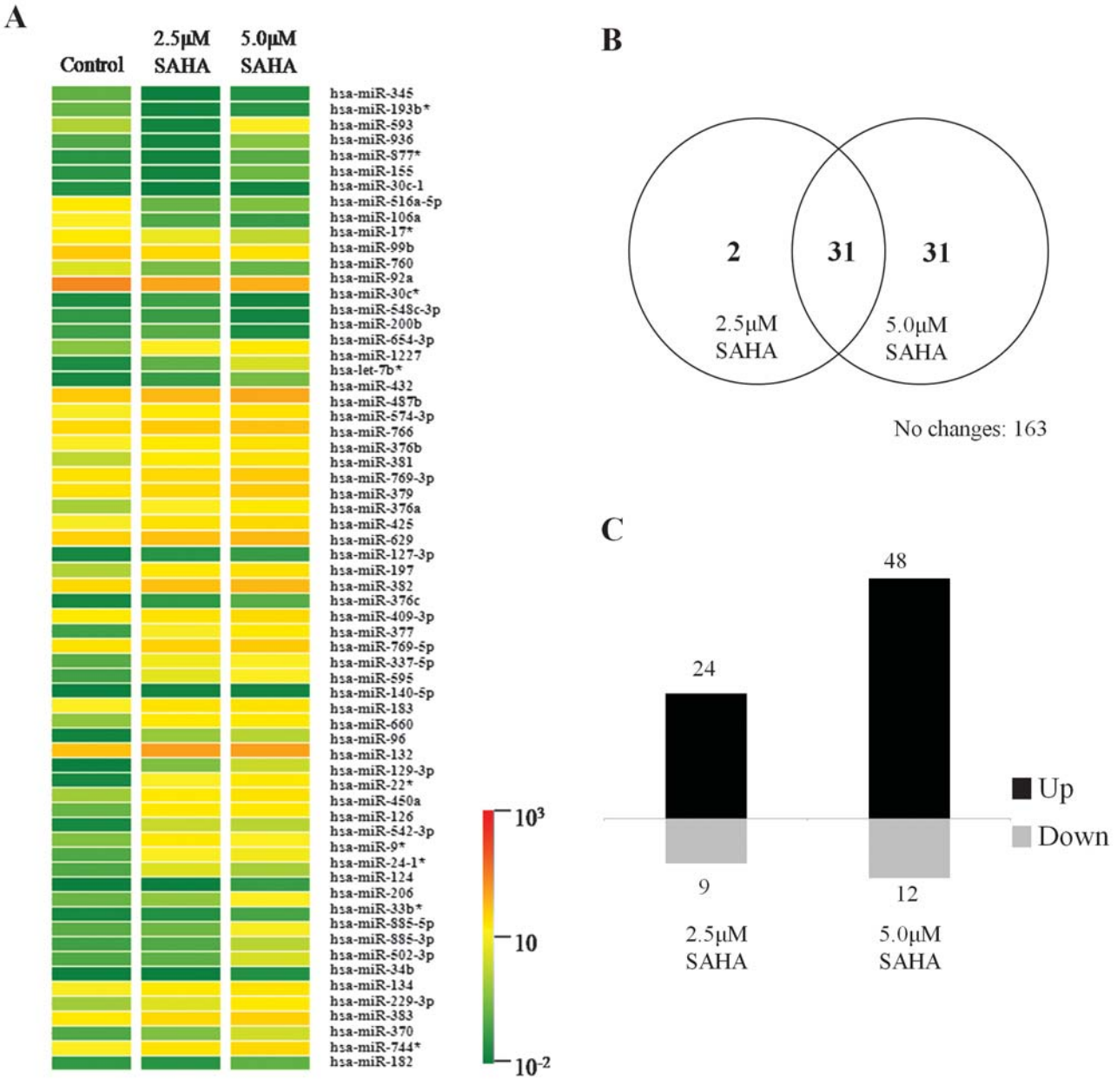

Figure 2. miRNA expression profiles that showed >2-fold change in comparison with each group after SAHA treatment. Selected miRNAs and their expression levels are indicated by color (A). The Venn diagram shows the expression patterns of several miRNAs that are influenced by SAHA dose. Altogether, 31 miRNAs are changed in both conditions (B). Among 64 miRNAs showing 2-fold change, the miRNAs demonstrate up- or down-regulation in each treatment. The number of miRNAs with increased or decreased expression in each treatment (C).

Table I. 64 miRNAs showing 2-fold expression changes after SAHA exposure.[a]

	miRNA profiles					
	Up				Down	
Concentration of SAHA (μM)	miRNA name	Fold change	miRNA name	Fold change	miRNA name	Fold change
2.5	hsa-let-7b*	4.18	hsa-miR-376a	2.26	hsa-miR-106a	3.81
	hsa-miR-1227	3.93	hsa-miR-382	3.55	hsa-miR-155	5.38
	hsa-miR-124	5.79	hsa-miR-409-3p	4.2	hsa-miR-193b*	17.98
	hsa-miR-126	12.39	hsa-miR-450a	3.61	hsa-miR-30c-1*	11.8
	hsa-miR-127-3p	2.48	hsa-miR-542-3p	2.74	hsa-miR-345	27.51
	hsa-miR-129-3p	22.6	hsa-miR-595	2.1	hsa-miR-516a-5p	3.65
	hsa-miR-132	34.02	hsa-miR-629	2.63	hsa-miR-593	15.67
	hsa-miR-183	2.54	hsa-miR-654-3p	2.08	hsa-miR-877*	10.32
	hsa-miR-22*	2.76	hsa-miR-660	26.58	hsa-miR-936	12.91
	hsa-miR-24-1*	2.93	hsa-miR-769-5p	2.9		
	hsa-miR-30e*	2.71	hsa-miR-9*	3.86		
	hsa-miR-337-5p	3.52	hsa-miR-96	2.19		
5.0	hsa-let-7b*	9.29	hsa-miR-379	2.6	hsa-miR-106a	5.69
	hsa-miR-1227	8.47	hsa-miR-381	2.4	hsa-miR-17*	2.3
	hsa-miR-124	225	hsa-miR-382	6.35	hsa-miR-193b*	2.45
	hsa-miR-126	11.46	hsa-miR-383	2.07	hsa-miR-200b	2.79
	hsa-miR-127-3p	3.7	hsa-miR-409-3p	7.71	hsa-miR-30c-1*	7.36
	hsa-miR-129-3p	33.95	hsa-miR-425	2.12	hsa-miR-30e*	4.62
	hsa-miR-132	55.34	hsa-miR-432	2.24	hsa-miR-345	2.77
	hsa-miR-134	2.02	hsa-miR-450a	3.63	hsa-miR-516a-5p	3.08
	hsa-miR-140-5p	2.01	hsa-miR-487b	2.3	hsa-miR-548c-3p	4.95
	hsa-miR-182	2.09	hsa-miR-502-3p	2.73	hsa-miR-760	2.09
	hsa-miR-183	2.76	hsa-miR-542-3p	2.44	hsa-miR-92a	2.03
	hsa-miR-197	2.5	hsa-miR-574-3p	2.07	hsa-miR-99b	2.16
	hsa-miR-206	2.64	hsa-miR-595	2.19		
	hsa-miR-22*	3.25	hsa-miR-629	3.57		
	hsa-miR-24-1*	2.25	hsa-miR-654-3p	3.15		
	hsa-miR-299-3p	2.26	hsa-miR-660	33.41		
	hsa-miR-337-5p	4.84	hsa-miR-744*	2.7		

[a]The list was selected to show only those miRNAs representing >2-fold expression change after flag sorting.

separated into four groups: angiogenesis (23 genes), apoptosis (178 genes), chromatin modification (20 genes) and cell proliferation and differentiation (75 genes). The overlapping genes that were acquired through the comparison between those from the miRBase Target and those from the Gene Ontology are listed in Table II. It is important to note that each miRNA has several target mRNAs, and each target could be controlled by many miRNAs (30). In this analysis, all miRNAs in the list have at least one of their predicted target genes related to the four groups, and some of them have more than one target gene. Among 23 angiogenic genes identified by Gene Ontology, miRBase Targets predicted 14 genes that could be regulated by 17 of the candidate miRNAs. We found 94 genes associated with apoptosis, six genes associated with chromatin modification and 23 genes associated with cell proliferation and differentiation.

In conclusion, our data indicate that SAHA treatment of A549 cells causes changes in the miRNA expression profile. Although SAHA has been successfully shown to be an effective anticancer agent, the mechanism by which this occurs is not fully understood. However, this study shows that certain miRNAs undergo changes in response to SAHA and may differentially regulate their target genes, which may further clarify the role of SAHA in cancer therapy.

miRNA target genes dependent on concentration of SAHA from Table I.

miRNA name	Functions of target genes			
	Angiogenesis	Apoptosis	Chromatin modification	Cell proliferation and differentiation
hsa-let-7b*	EPGN, MYH9, SCG2	BFAR, CHEK2, DLC1, FASLG, HBXIP, PMAIP1, SAP30BP, SCG2, SON, TP53BP2	INOC1	DLC1
hsa-miR-106a	RNH1	BAX, BCL2L11, BNIPL, HBXIP, NUP62, P2RX4, PPP3R1, TNFRSF10B	-	BNIPL, NUP62
hsa-miR-1227	N/A	N/A	N/A	N/A
Hsa-miR-124	HTATIP2, SPHK1	AGT, APOE, BAX, CADM1, CD27, ERCC3, GRIK2, HTATIP2, RELA, SIVA1, TGFB1, TNFSF15	-	-
hsa-miR-127-3p	-	AIFM3, BAD, BCL6, NLRP1, PPP3CC	HTATIP	ING5
hsa-miR-129-3p	-	ANGPTL4, BAD, CASP8, CDKN1B, CDKN2C, CRYAB, DEDD2, ERCC3 IFI16, ING4, PPP3R1, SFN, STK4, TP53	-	-
hsa-miR-132	AMOT	BCL2L11, CASP8, GHRL, HIP1, IL6, LCK, PEA15, PML, RASA1, YWHAB	TNP1, PML	APC, ENPEP, ENPP7
hsa-miR-183	-	CASP10, DFFB, GRIK2, HIP1, IL12B, LCK, PPP3CC, SRA1, TCF7L2	HDAC10	BTG1, EPO, RASGRP4, SRA
hsa-miR-22*	AGGF1, HHEX	AIFM3, CASP8, CIDEC, FASLG, IL6, RTKN, TP53BP2, TRADD	-	EDN2
hsa-miR-337-5p	-	AGT, ANGPTL4, BCL10, HBXIP, HSPA5, SFN, TCF7L2, TP53BP2	-	-
hsa-miR-376a	-	CADM1, CASP8, FAS, HBXIP, IL6, PPP3CC, TERF1	-	GLI2, PCAF
hsa-miR-382	-	ADAMTSL4, CD40LG, CDKN2C, GLO1, HSPD1, IFI16, IFNG, PPP3CC	RSF1	CDKN2C, DERL2, MYC, RERG
hsa-miR-409-3p	COL4A3, NCL	AKT1, COL4A3, DNAJB6, ERCC3, TCF7L2	-	EPO
hsa-miR-629	ANGPTL3, COL4A3, EPGN	ALB, APOE, COL4A3, DLC1, GRIK2, RTKN, TCF7L2	-	DLC1, ENPEP MYC, POLA1
hsa-miR-654-3p	-	ACIN1, CASP10, CDKN2A, NDUFA13, P2RX4, RPS3, TNF	-	-
hsa-miR-660	-	ADAMTSL4, CADM1, CECR2, HIP1, IFI16, IFT57, LCK, LGALS12, UBE4B	-	RERG
hsa-miR-769-5p	-	BCL2L11, CRYAA, ING4, NDUFS1, NLRP1, PCBP4, RELA, TBX3	-	EPO, GLI2, ING4, MEN1, TBX3
hsa-miR-96	IL17F, NCL, PROK2	AIFM3, CRYAB, DYNLL1, GRIK2, LCK, P2RX4, PCBP4, PROK2, SON, SRA1	HDAC10, HTATIP	PROK2, SRA1

N/A, not applicable.

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