SPANDIDOS PUBLICATIONS

Gene expression profiling induced by histone deacetylase inhibitor, FK228, in human esophageal squamous cancer cells

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Abstract. Histone deacetylase inhibitors (HDACIs) are promising therapeutic agents with the potential for regulating cell cycle, differentiation and apoptosis in cancer cells. HDACI activity is associated with selective transcriptional regulation and altering gene expression. However, the exact mechanisms leading to the antitumor effect of HDACIs are not fully understood. FK228, one of the powerful HDACIs, strongly inhibited cell growth of T.Tn and TE2 cells and induced apoptosis. Therefore, comprehensive analysis of the changes in gene expression in human esophageal cancer cell lines by the HDACI FK228 was carried out by microarray analysis. This analysis was used to clarify the expression profiles of genes after exposure to FK228. Of the 4,608 genes analyzed, 93 genes in T.Tn and 65 genes in TE2 were up- or down-regulated 2-fold or more at least at one time point during FK228 exposure and they were classified into four clusters based on their expression patterns. Among them, 15 genes were contained in both cell lines and their expression patterns were similar. Except p21, Prdx1 (reported by us) and IGFBP3, the behaviour/expression of 12 highly responsive genes has still not been reported in esophageal cancer cells. These observations of the expression patterns of functionally classified genes provided insights into the mechanism of the antitumor effect of FK228 in esophageal squamous carcinoma.

Introduction

Esophageal squamous cell carcinoma (ESCC) remains one of the intractable cancers due to its higher incidence in older patients and its rapid infiltration into neighboring tissues despite the recent progress in multi-modal approaches (1,2). Currently, cisplatin and 5-fluorouracil are considered the optimal chemotherapy agents for ESCC (3). However, the response rates of these combination chemotherapies are not satisfactory and the development of new anticancer drugs is expected to improve the prognosis of ESCC. Histone deacetylase inhibitors (HDACIs) are considered to represent one of the most promising agents for this purpose.

The balance of acetylation by histone acetylases and deacetylation by histone deacetylase prevents full acetylation, thereby creating a default underacetylated state. Acetylated histone tails and other chromosome-associated proteins have been identified in nucleosomes and shown to be important in regulating gene expression (4,5). Histone acetylation is often associated with the transcription of gene characteristics of the differentiated state. In contrast, histone deacetylation correlates with transcriptional silencing and specifically, down-regulation of the expression of pro-apoptotic genes. The anticancer effects of HDACIs were mainly thought to be activated by the modulation of gene expression patterns including genes associated with cell cycle arrest and apoptosis by inhibiting HDAC activities. The induction of apoptosis by HDACIs via cell death pathways mediated by TRAIL, Fas, Bid and p53 was also reported (6-10). However, a comprehensive analysis of the expression profiles of these genes has not been reported.

FK228, one of the novel histone deacetylase inhibitors and isolated from *chromobacterium violaceum*, has exhibited remarkable antitumor activity in xenograft models and patients with cutaneous T-cell lymphoma had a complete or partial response in a phase I trial (11,12). The current study was designed to explore the efficacy of the antitumor activity on human esophageal squamous cancer cell lines and to identify and characterize additional target genes of FK228. By using microarray analysis, we revealed gene expression profiles during the exposure of FK228 for up to 48 h. Ninety-three genes in T.Tn and 65 genes in TE2 were shown to be regulated during the course of FK228 exposure. Among them, 15 genes showed substantial induction in both cell lines and their microarray analysis gave insights into the roles and regulation of genes by FK228.

Materials and methods

Assay of cytotoxicity. Cell growth was determined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). $5x10^3$ cells per well were seeded into 96-well microplates and incubated for

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48 h at 37˚C under 5% CO_2. FK228 was dissolved in ethanol and diluted with DMEM. After changing the medium, test samples were changed and incubated in the presence or absence of drugs for 72 h. Then the Cell Counting Kit-8 reagent was added and allowed to react for 3 h. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The IC50 (50% inhibitory concentration) values were calculated by the least squares method.

mRNA preparation and cDNA microarray analysis. Cells were seeded into 225-cm^2 flasks and incubated for 48 h, then treated with or without IC80 concentration of FK228 for up to 48 h and harvested at 0, 6, 12, 24 and 48 h. Cells were washed with PBS and processed for RNA extraction with the RNeasy Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Total RNA (20 μg) from cells cultured with FK228 was compared with 20 μg of total RNA from cells cultured without FK228, using the cDNA microarray, which consisted of 4608 distinct cDNA clones, generated as described previously (13). Fluorescent images of the hybridized microarrays were scanned with a fluorescence laser confocal slide scanner (Scan Array 4000, GSI Lumonics, Nepean, Canada) and analyzed with Quant Array software (GSI Lumonics, Nepean, Canada) according to the manufacturer's instructions. All experiments were done in duplicate and averaged data for each of the time points were subjected to statistical analysis. To identify genes significantly expressed, we defined the data for those genes that showed 2-fold changes for at least one time point.

Real-time quantitative RT-PCR. The expression changes of 5 randomly selected genes (prohibitin, S100A6, ZNF24, EIF4A1, cyclin I) were also examined by real-time quantitative PCR using the Light Cycler technique (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA templates for real-time PCR were synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase and an oligo-dT primer. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an internal control. The PCR reaction consisted of DNA Master SYBR-Green I mix (Light Cycler-FastStart DNA Master SYBR-Green I kit, Roche Diagnostics; containing Taq DNA polymerase, dNTP, 3 mM $MgCl_2$ and SYBR-Green dye), 0.5 μM each primer and cDNA. The PCR processes were as follows: initial denaturation at 95˚C for 10 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, annealing at 57˚C for 10 sec and elongation at 72˚C for 8-18 sec. The Fit Points method provided by the Light Cycler software was used to estimate the concentration of each sample. Primers were chosen using Primer 3 (available at: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The following primer sequences were used: Prohibitin: 5'-CAGCATCGGAGAGG ACTATGAT-3' and 5'-GAAGGTCAGATGTGTCAAGGA C-3'; S100A6: 5'-GTTGCTTTGAGAGGAGGACACT-3' and 5'-CCCCATAGATGATGAGAGTGGT-3'; ZNF24: 5'-A CCCCAAAGAGCTACAGACTTGG-3' and 5'-CAGAACTT GGTAATCCCTGAGC-3'; EIF4A1: 5'-TGCTTAACCGGA GATACCTGTC-3' and 5'-GTCCCTCATGAACTTCTTGG TC-3'; cyclin I: 5'-GTCAGAGGTACAGCAGCCTTTT-3' and 5'-GAAGCATGTCCCTCTTGTCTTG-3'; GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCC TGTTGCTGTA-3'. The expression value was calculated as follows: expression level of each mRNA/expression level of GAPDH.

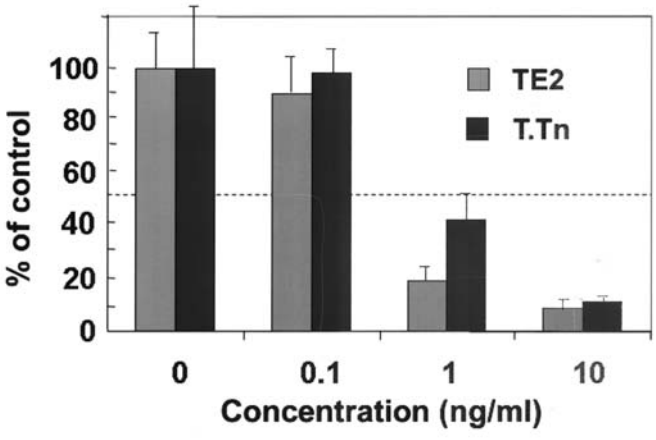

Figure 1. Effect of FK228 on human esophageal squamous cancer cell lines, TE2 and T.Tn. Cells were incubated for 72 h in the absence or presence of the indicated concentrations of FK228. Then, the growth inhibitory effect of FK228 was examined by Cell Counting Kit-8 as described in Materials and methods. Data are presented as a percentage of viability in control ± SD (n=8).

Annotation of gene function. The normalized Cy5/Cy3 ratios in the microarray analysis were log2-transformed and used to classify the patterns of serial changes of the gene expression. Genes whose expression levels varied at least 2-fold at any of the time points were subjected to a hierarchical clustering analysis using the algorithm of Euclid and Ward in GeneMaths software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

The molecular functions of the genes were assigned by referring to Gene Ontology™ (www.geneontology.org/) and GeneCards™ (bioinfo.weizmann.ac.il/cards/).

Results

FK228 demonstrated anti-proliferative activity by inducing apoptosis in human esophageal cancer cell lines. FK228 has apparent anti-proliferative activity against human cancer cells (6-10,14). First, the effect of FK228 on cell growth was determined in the human esophageal cancer T.Tn and TE2 cells. A lower dose (IC50 values were 0.373 and 0.781 ng/ml, respectively) of FK228 produced changes in growth rate compared to control cells at 72 h in both T.Tn and TE2 cell lines (Fig. 1). In addition, our previous data (14) showed that FK228 significantly induced apoptosis.

Application of cDNA microarray analysis for detection of changes in gene expression by exposure to FK228. To examine the sequential changes in gene expression upon treatment of esophageal cancer cell lines with FK228, we performed cDNA microarray analysis. Total RNAs (20 μg each) derived from cells at 6, 12, 24, or 48 h during FK228 exposure and from control cells in duplicate were subjected to

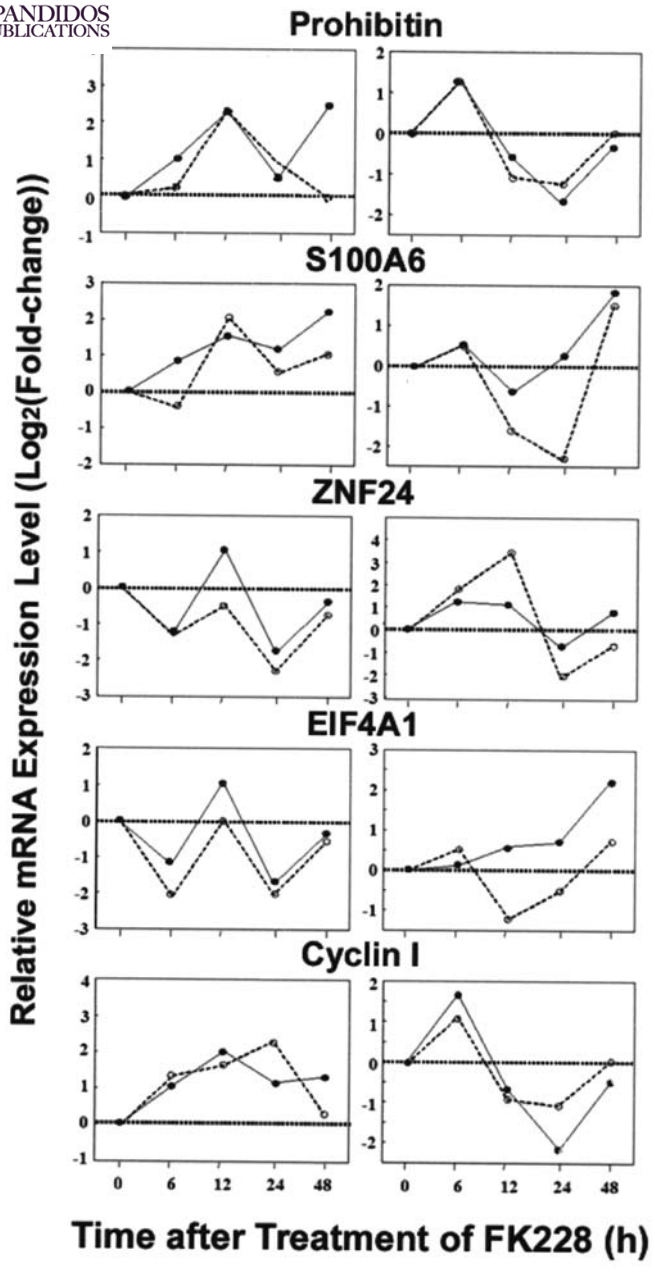

Figure 2. Comparison of results obtained with microarray and real-time quantitative reverse transcription-PCR for changes in mRNA levels after FK228 treatment. mRNA levels at various time points after FK228 treatment compared to the levels of non-treatment cells in microarray analysis (closed circles) and real-time quantitative reverse transcription-PCR analysis (open circles) are shown for the following mRNAs: prohibitin, S100A6, ZNF24, EIF4A1 and cyclin I.

Cy3 and Cy5 labeling, respectively, coupled with cDNA synthesis. Both cDNAs were mixed in equal amounts and hybridized with a microarray. Out of the 4,608 genes analyzed, we regarded the expressions of 95 genes in T.Tn and 63 genes in TE2 as meaningful, because their expression levels were altered at least 2-fold either up or down at certain points of the time course.

To confirm the validity of the results obtained by the microarray analysis, 5 randomly selected genes were examined by real-time quantitative RT-PCR analysis. The mRNA levels of these genes were nearly comparable between microarray and real-time quantitative RT-PCR, generally verifying the competency of the microarray analysis for detection of changes in gene expression during exposure to FK228 (Fig. 2).

Serial changes in expression levels of genes in relation to their functions. The 95 genes in T.Tn and 63 genes in TE2 with changes in their expression levels after FK228 exposure were subjected to clustering analysis based on their expression patterns and were classified into four clusters (Fig. 3A and 4A). The time course of changes in mRNA levels in each cluster can be roughly characterized as follows: cluster A, general decrease; cluster B, gradual increase; cluster C, rapid increase and persistence; cluster D, transient up-regulation and gradual decrease (Fig. 3B and 4B).

We also categorized these genes based on their functions, referring to Gene Ontology™ and classified them into seven groups: apoptosis, cell cycles, defense and immunity, metabolism, signal transduction and transcription, structural protein and unclassified. The frequencies of these functionally classified genes in each cluster are shown in Fig. 3C and 4C.

A comparison of gene expression patterns in the two cell lines showed that 11 genes were up-regulated and 4 genes were down-regulated in both cell lines (Table I and II). Not all of these genes were classified into the same clustering groups, but fundamentally their expression patterns were similar, especially of the down-regulated 4 genes that were included in the same group.

Discussion

HDACIs, such as FK228, have become known as potent inducers of differentiation and apoptosis in cancer cells (6,7). However, the mechanisms of growth inhibition by HDACIs have not been well characterized. In this study, we examined the *in vitro* and *in vivo* antitumor effects and the modulation of gene expression in human esophageal squamous cancer cells by FK228.

We showed that FK228 induces growth inhibition and apoptosis on the human esophageal cancer cell lines T.Tn and TE2. FK228 strongly inhibited the growth of T.Tn and TE2 cells *in vitro*. According to our previous data, a modest induction of apoptosis was observed after FK228 treatment (14).

Activities of HDACIs are required at least in part for transcriptional modulation, as this therapeutic approach is described as 'transcription therapy' (15). However, the molecular events that correlate with growth inhibition and apoptosis induction by HDACIs still remain to be proven.

The development of microarray technology has facilitated the analysis of the expression of thousands of genes in a single experiment (16,17). In the present study, we used microarray analysis to find gene expression changes in response to FK228. We identified several genes that significantly up-regulated in both T.Tn and TE2 cells and some of those genes have been reported as being related to cell cycle arrest or tumor suppression. The 15 genes significantly changed in both cell lines consisted of 11 up-regulated genes and 4 down-regulated genes.

Cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$ was clearly up-regulated in our data and it has been reported to

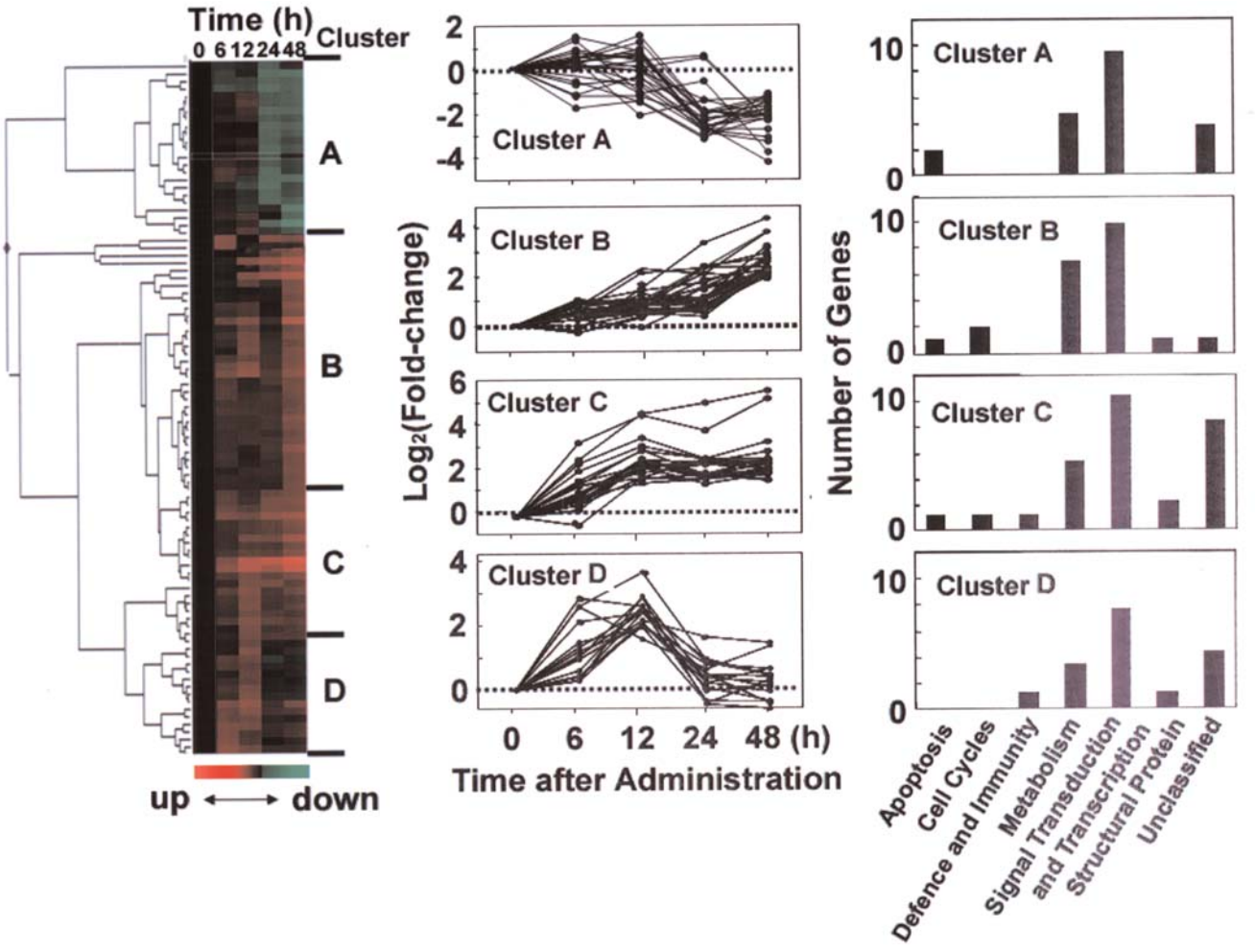

Figure 3. Gene expression profiles after FK228 treatment in T.Tn cells. (A) Cluster analysis of genes with changed expression levels after FK228 treatment. mRNA levels were assessed at 6, 12, 24, and 48 h after treatment. A total of 93 genes whose intensities varied 2-fold or more at least at one time point were subjected to a hierarchical clustering analysis. Time points are shown in columns and genes in rows. Light red, black and light green represent the higher, equal and lower mRNA levels relative to that of the control cells, respectively. These 93 genes were classified into 4 clusters (Cluster A to D) by using GeneMaths software. (B) Expression patterns of genes in each cluster. mRNA levels of each gene at various time points after FK228 treatment relative to control mRNA levels (dotted line) in non-treatment cells are shown. (C) The distribution of functionally categorized genes in each cluster. Frequencies of genes of each functional category are shown.

play an important role in growth arrest, both in G1 and G2/M cell cycle arrest (18). Similar to our previous results with human esophageal cancer cell lines (14), other studies revealed that HDACIs induced cell cycle arrest by regulating $p21^{WAF1}$ in some other cell lines (19,20).

Peroxiredoxins (Prdx) are a novel family of 25 kD peroxidases that can reduce H_2O_2 using an electron donor from thioredoxin and/or glutathione (21). Recently, Prdx1 was reported to inhibit c-Abl kinase activity and to regulate certain c-Myc-dependent functions and the abilities suggested that Prdx1 was a candidate tumor suppressor gene (22,23). Neumann *et al* (24) had generated Prdx1-lacking mice and malignancies including digestive cancers, lymphomas and sarcomas were seen in them at a high frequency. In fact, our previous data demonstrated that the induction of Prdx1 was correlated with the chemosensitization by FK228 (14).

EPB41, protein 4.1R (red blood cell type), has been identified as an 80 kD protein that plays a structural role in the membrane skeleton of human erythrocytes (25). Moreover, recent studies suggest that EPB41 may have a function as a tumor suppressor. Robb *et al* demonstrated a loss of EPB41 expression in 40% of meningioma patients and the over-expression of EPB41 resulted in a reduction of meningioma cell proliferation (26). There are no reports on any relationship between EPB41 and esophageal cancer.

HSPA1A is one of the members of the HSP70 family that is augmented by stress response, which occurs as a result of a variety of environmental cues including heat shock, heavy metals, oxidative stress, inflammation and others (27). Rohde *et al* (28) found that cancer cells depleted of HSP70 and HSP70-2 (HSPA2) by small interfering RNA displayed strikingly different morphologies (detached and round vs flat senescent-like), cell cycle distribution (G2/M vs G1 arrest), and gene expression profiles. Concomitant depletion of HSP70 and HSP70-2 had a synergistic anti-proliferative effect on cancer cells. In our microarray analysis, HSPA1A expression changes were transient up-regulation and gradual decrease in both cell lines, indicating that the alteration in HSPA1A may occur as a result of the stress response by FK228.

PTRF, PolI and transcript release factor, was initially identified as a complementation of a release-deficient cellular PolI for transcript release (29). Jansa *et al* (30) demonstrated

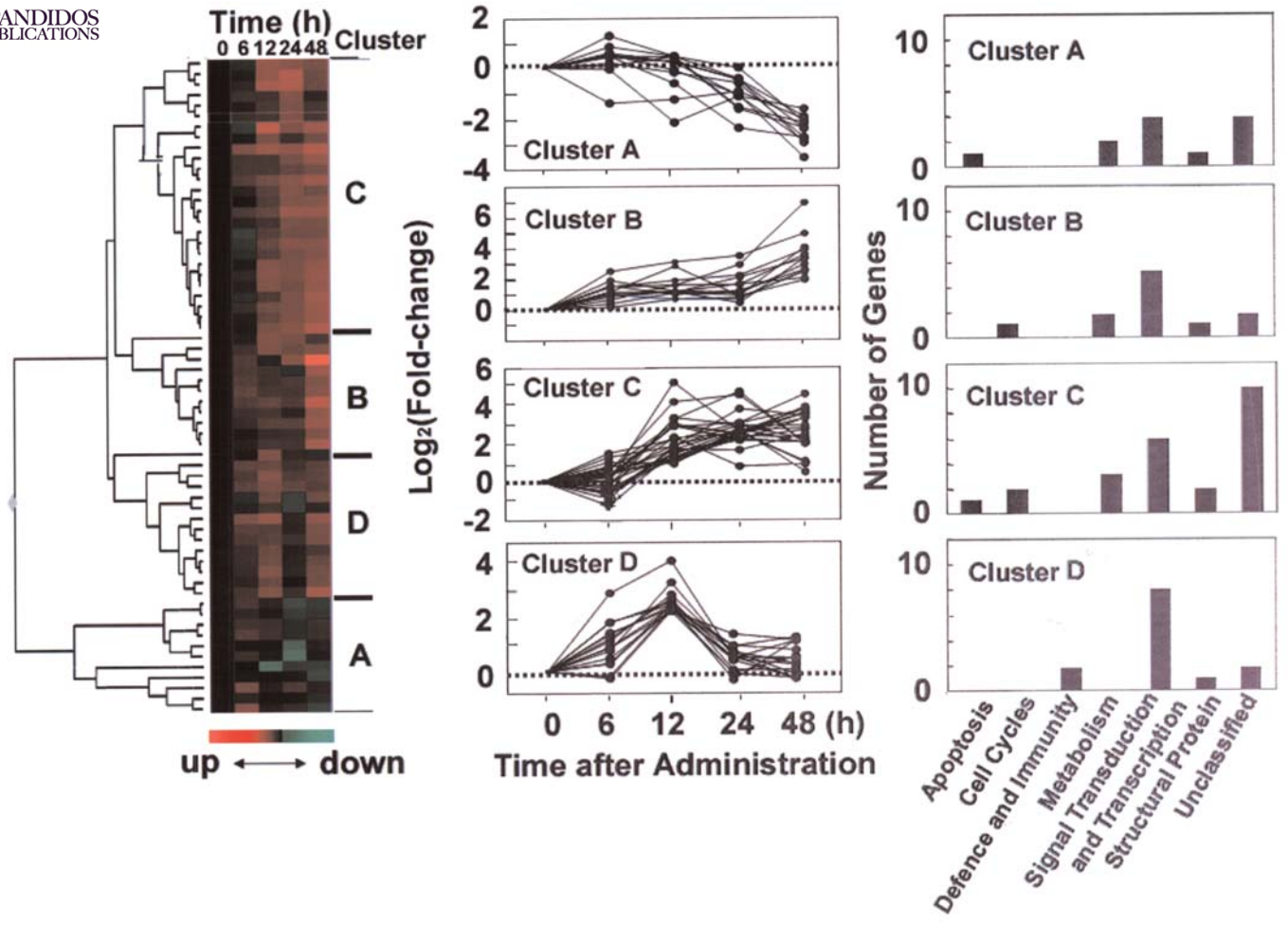

Figure 4. Gene expression profiles after FK228 treatment in TE2 cells. (A) Cluster analysis of genes with changed expression levels after FK228 treatment. mRNA levels were assessed at 6, 12, 24, and 48 h after treatment. A total of 65 genes whose intensities varied 2-fold or more at least at one time point were subjected to a hierarchical clustering analysis. Time points are shown in columns and genes in rows. Light red, black and light green represent the higher, equal and lower mRNA levels relative to that of control cells, respectively. These 65 genes were classified into 4 clusters (Cluster A to D) by using GeneMaths software. (B) Expression patterns of genes in each cluster. mRNA levels of each gene at various time points after FK228 treatment relative to control mRNA levels (dotted line) in non-treatment cells are shown. (C) Distribution of functionally categorized genes in each cluster. Frequencies of genes of each functional category are shown.

that PTRF interacts with both terminator proteins, TTF1 and PolI and binds to transcripts containing the 3-prime end of pre-rRNA *in vitro*. Recombinant PTRF induced the dissociation of ternary PolI transcription complexes *in vitro*, releasing both PolI and nascent transcripts from the template. In the situation of activating the transcription by HDACI, PTRF might be up-regulated to regulate transcriptional termination. The mechanisms of this gene in esophageal cancer cells have not been clarified.

GRN, progranulin, is a 593-amino acid glycoprotein, the mRNA of which is expressed by many epithelial cells both *in vitro* and *in vivo*. He *et al* (31) showed that the over-expression of the progranulin gene in adrenal carcinoma cells and nontransformed renal epithelia cells resulted in transfection-specific secretion of progranulin, acquired clonogenicity in semisolid agar and increased mitosis in monolayer culture. Progranulin is also cleaved into its component granulins, small proteins of 6 kD and these proteins have been shown to have an inhibitory function, opposing that of progranulin. Although we have not demonstrated the role of progranulin in esophageal cancer cells with the exposure to HDACI, it might be related to the inhibition of cell growth.

PSAP, prosaposin, is a glycoprotein encoded by a single locus on human chromosome 10 and is the precursor of four sphingolipid activator proteins named saposins A, B, C and D that are localized within lysosomes and that activate the hydrolysis of sphingolipids by lysosomal hydrolases (32). In PC12 pheochromocetoma cells, prosaposin was able to activate extracellular signal-regulated protein kinases (ERKs) and sphingosine kinase (SK) with sphingosine-1-phosphate production, thus eliciting an effect of proliferation and cell death prevention (33). Prosaposin was classified into cluster B (gradual increase, Fig. 3B and 4B) meaning that it was not affected by FK228 directly, but it might be affected in some indirect manner through another protein induced by FK228.

MTATP8, ATP synthase 8, is encoded by nucleotides of the mitochondrial genome. Mitochondria play a pivotal role in providing energy for cells and in the execution of apoptosis (34). However, the role of mitochondria in cancer is not clear. Recently, Warburg's hypothesis was supported by studies that provided a pathway by which cancer cells down-regulate

Table I. List of 11 commonly up-regulated genes with at least two-fold change.

No.	Accession no.	Symbol	Title	Function	Cluster (T.Tn/TE2)	Log_2 ratio after the indicated time (h) of FK228 treatment (T.Tn/TE2) 6	12	24	48
1	U03106	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Cell cycle	C/B	2.25/1.98	3.41/2.54	2.32/2.87	3.11/3.75
2	X67951	Prdx1	Peroxiredoxine 1	Metabolism/enzyme	B/C	0.52/0.44	1.05/1.98	2.24/2.11	3.85/4.02
3	M61733	EPB41	Erythrocyte membrane protein band 4.1	Cytoskeleton	B/B	0.43/0.98	0.87/1.43	1.12/1.67	2.22/2.53
4	M59828	HSPA1A	Heat Shock 70 kDa protein 1A	Transcription factor	D/D	0.87/2.98	2.95/3.74	0.03/0.76	0.01/0.12
5	AK000715	PTRF	Polymerase I and Transcript release factor	Translation factor	B/B	0.34/0.45	0.98/1.32	1.21/1.82	2.20/3.24
6	X62320	GRN	Granulin	Translation factor	C/B	1.23/1.44	3.05/1.98	2.87/2.11	2.65/3.70
7	AL132826	FLRT3	Fibronectin leucine-rich transmembrane protein 3	Signal transduction	B/D	0.42/0.88	0.89/3.01	2.24/0.78	2.51/0.44
8	M81355	PSAP	Prosaposin	Signal transduction	B/B	0.51/2.23	0.92/2.64	1.32/2.97	2.73/4.52
9	V00662	ATP8	ATP synthase 8	Metabolism/enzyme	C/B	0.48/0.78	0.97/1.11	1.21/1.24	2.22/2.70
10	X93334	ND4L	NADH dehydrogenase 4L	Metabolism/enzyme	D/C	0.48/0.64	2.24/1.82	0.46/2.11	0.52/3.70
11	BC003560	RPN2	Ribophorin II	Metabolism/enzyme	D/B	0.89/0.56	3.30/0.98	0.52/1.17	0.71/2.36

Table II. List of 4 commonly down-regulated genes with at least two-fold change.

No.	Accession no.	Symbol	Title	Function	Cluster (T.Tn/TE2)	Log_2 ratio after the indicated time (h) of FK228 treatment (T.Tn/TE2) 6	12	24	48
1	BC000013	IGFBP3	Insulin-like growth factor binding protein 3	Regulation of cell growth	A/A	-1.43/-0.13	-0.98/-2.10	-1.88/-1.24	-2.46/-1.78
2	BC005391	RPSA	Laminin receptor 1 (ribosomal protein SA, 67 kDa)	Regulation of transcription	A/A	1.29/-0.13	-1.61/0.23	-2.29/-1.87	-1.00/-2.25
3	D89092	HNPDL	Heterogeneous nuclear ribonucleoprotein D-like	Translation factor	A/A	0.59/-0.08	-1.16/0.28	-2.41/-1.27	-1.94/-2.99
4	BC002355	HNRPA1	Heterogeneous nuclear ribonucleoprotein A1	Translation factor	A/A	0.54/0.54	-0.19/0.12	-2.56/-1.14	-1.72/-3.65

mitochondrial activity (35). In kidney, colon, squamous esophageal cancer, as well as in lung, breast and gastric adenocarcinomas, expression of the ATP synthase was lower, consistent with selective repression of the expression of components involved in mitochondrial bioenergetic function. Shin *et al* (36) reported that ATP synthase down-regulation might lead to cellular events responsible for 5-FU resistance, so our findings may be concerned with the chemosensitization of FK228.

Fibronectin-like domain-containing leucine-rich transmembrane protein 3 (FLRT3), was localized to presynaptic axon terminals (37). Recently, Bottcher *et al* (38) found that FLRT3 was a novel modulator of FGF signaling. FLRT3 is a transmembrane component of the FGF signaling pathway, whose expression is regulated by FGFs and which promotes canonical FGF signaling through the MAPK pathway that is blocked by MAPK phospatase 1. FLRT was classified into cluster B or D, so this molecule might play some roles in MAPK signal transduction.

ND4L, NADH dehydrogenase 4L, is one of the 7 mitochondrial DNA-encoded subunits included among ~ 41 polypeptides of respiratory complex I (39). Interest in

dria with regard to neoplasia has revived, because ole in apoptosis and other aspects of tumor biology. Polyak *et al* (40) found a 10563T-C transition resulting in a cys32-to-arg amino acid substitution in the MTND4L protein in colorectal cancer. Up-regulation of ND4L by FK228 might possibly be correlated with the efficacy of this drug.

Ribophorin II, one of the specific glycoproteins, spans the rough regions of the endoplasmic reticulum (RER) and is thought to play an important role either in translocation or in the maintenance of RER (41). The mammalian oligosaccharyltransferase (OST) is a protein complex composed of four rough ER-specific, type I transmembrane proteins: ribophorins I and II, OST48 and DAD1 (defender against cell death 1) (42). The DAD1 protein has been found to permit temperature-sensitive cells to escape from apoptosis. Therefore, the overexpression of one component of OST might be related to inhibiting apoptosis by this agent in esophageal cancer cells.

One of the down-regulated genes, insulin-like growth factor binding protein 3 (IGFBP3), was originally defined by the somatomedin hypothesis as the principal carrier of IGF-I in the circulation and the primary regulator of the amount of free IGF-I available to interact with the IGF-I receptor (43). However, there is accumulating evidence that IGFBP3 can also cause apoptosis in an IGF-independent manner or p53-dependent and -independent fashion. However, interestingly, in some colon cancer cell lines, including SW480 and SW948, IGFBP3 also induces proliferation (44). Moreover, it has been reported that IGFBP3 mediates the TGF-ß 1 proliferative response in the metastatic or highly aggressive colon cancer cell line (45). Furthermore, IGFBP3 mRNA was overexpressed in primary esophageal squamous cell carcinoma (46). From these results, the molecular functions of IGFBP3 are various and controversial. The down-regulation of IGFBP3 by FK228 should contribute to inhibiting proliferation in esophageal squamous cell carcinoma. We are currently examining the precise function of IGFBP3 in esophageal cancer cells.

Heterogeneous nuclear ribonucleoprotein D-like protein (HNRPDL) shuttles between the nucleus and the cytoplasm and the shuttling protein can interact directly with mRNA (47). Under the transcription inhibition conditions resulting in HNRPDL cytoplamic accumulation, the HNPRDL-Poly (A)+ RNA complex was found at higher levels in the cytoplasm than in the nucleus, whereas under transcriptional conditions, this complex was found in the nucleus, but not in the cytoplasm. These results suggest that shuttling HNPRDL may carry mRNA from the nucleus to the cytoplasm. However, our results indicated that HNPRDL was down-regulated and it may be unclear and even paradoxical, why HNPRDL was down-regulated under transcriptional conditions by FK228.

Heterogeneous nuclear ribonucleoprotein A1 (HNRPA1) was reported to shuttle continuously between the nucleus and cytoplasm and that it contains a 38-amino acid domain that acts as both a nuclear localization and nuclear export signal (48). These authors suggested that HNRPA1 functions as a carrier for RNA during export to the cytoplasm. Chiu *et al* (49) demonstrated by cDNA microarray and real-time RT-PCR that HNRPA1 was up-regulated in colon cancer patients. Furthermore, Wei *et al* (50) also showed that HNRPA1 was significantly up-regulated in refractory acute leukemia patients. Presumably, HNRPA1 plays a role in the positive regulation of cancer development. Thus, down-regulation of HNRPA1 by FK228 may be associated with the cytotoxic activities of FK228 in esophageal cancer cells.

Laminin receptor 1 (RPSA) is a non-integrin protein that is co-expressed and co-regulated with the α6 integrin subunit and is physically associated with this integrin on the cell membrane (51). Laminin is a basement membrane glycoprotein implicated in a large number of biologic activities of cancer progression, many of which are mediated by the presence of RPSA on the cell membrane. Expression of RPSA has been reported in a wide range of carcinomas, in many of which it was correlated with poor differentiation, metastasis, disease progression and poor survival (52). This indicates that the down-regulation of RPSA should contribute to the induction of the cytotoxic effect of FK228.

Our twelve genes, except p21, Prdx1 (as reported previously) and IGFBP3, have still not been reported in terms of their behaviour or expression in esophageal cancer cells. We are investigating each of the above genes, their expression and signal transduction in esophageal cancer cells, as well as their relation to the clinico-pathological findings of surgical specimens after esophagectomy. FK228 is one of the HDACIs currently under clinical development and these preliminary results from clinical trials suggest that this agent shows great promise (11,12). We are confident that our results of gene modulation profiling by FK228 in human esophageal squamous cells based on microarray analysis will lead to beneficial applications in the treatment of esophageal cancer and other carcinomas.

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