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# Marker genes to predict sensitivity to FK228, a histone deacetylase inhibitor

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**Abstract**

In this study, we detected genes sensitive to an histone deacetylase inhibitor, FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] in vitro and identified marker genes to predict sensitivity to FK228 in vivo using Affymetrix GeneChip. Three percent of genes (205/7070) were sensitive to FK228 in vitro, 105 and 100 genes, were up- and down-regulated, respectively, by FK228. Commonly up-regulated genes included $p21^{WAF1/Cip1}$, interleukin-8 (IL-8), histone family, JunB, caspase 9, mitogen-activated protein kinase phosphatase 1 (MKP-1) and mitogen-activated protein kinase (MAPK) family, and commonly down-regulated genes included cyclin A and MAPK family. One percent of genes (76/7070) showed native differences in patterns of expression, when FK228-sensitive (PC-3 prostate and SC-6-JCK (SC-6) stomach) and FK228-resistant (ACHN and A-498 renal) tumors implanted in BALB/c *nu/nu* mice were compared. Twenty-seven and forty nine of those genes were expressed at high or low levels, respectively, in FK228-sensitive tumors. Caspase 9 and MKP-1 genes showed distinct differences in patterns of expression between FK228-sensitive and resistant tumors and have been known to have roles in apoptosis and chromatin remodeling. The expression of caspase 9 gene was higher in FK228-sensitive tumors and the expression of MKP-1 gene was higher in FK228-resistant tumors. Caspase 9 and MKP-1 genes in the other FK228-sensitive tumors had the same patterns of expression as they did in PC-3 and SC-6 tumors. Our results present profiles of gene expression related to FK228 and marker genes to predict sensitivity to FK228, such as caspase 9 and MKP-1 genes.


*Keywords:* FK228; FR901228; Histone deacetylase inhibitor; Caspase 9; MKP-1; GeneChip

## 1. Introduction

FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraa zabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] is a unique bicyclic peptide containing a non-cystine disulfide bridge. It was first isolated from *Chromobacterium violaceum* strain WB968 as an agent inducing morphological reversion of H-ras transformed NIH 3T3 cells [1,2]. FK228 possesses potent antitumor activity against human tumor cell lines and significant inhibition effects on the growth of human solid tumors implanted in mice [3]. It has also been found to exhibit significant selectivity for tumor cells. Its $IC_{50}$ values against human tumor cells are of the order of several ng/mL and against human normal fibroblast cells >1000 ng/mL [3]. A recent study has shown that the growth-inhibition and apoptosis of oncogene-transformed cells induced by FK228 may involve blockage of the mitogen-activated signaling pathway, stimulation of a p53-independent expression of $p21^{WAF1/Cip1}$ and phosphorylation of Bcl-2 [4]. Recently, FK228 has been found to be a potent HDAC inhibitor, like TSA [5].

Several lines of evidence suggest that the histone acetylation plays a role in transcriptional regulation [6], probably by altering chromatin structure. Acetylation of core

*Abbreviations:* HDAC, histone deacetylase; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; SC-6, SC-6-JCK; LC-6, LC-6-JCK; nude, BALB/c *nu/nu*; MKP-1, mitogen-activated protein kinase phosphatase 1; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; CML, chronic myeloid leukemia; JNK, Jun-N-terminal kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; ERK, extracellular signal-related kinase

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nucleosomal histones is regulated by the opposing activities of histone acetyltransferases and HDACs [6]. HDACs catalyze the removal of an acetyl group from the e-amino group of lysine side chains of histone H2A, H2B, H3 and H4 [6]. Acetylation of histone proteins neutralizes the positive charge on lysine residues and disrupts nucleosome structure, leading to a loosening of histone-DNA contacts, access by transcription factor and change in gene expression [7].

HDAC inhibitors, such as FK228 [8] TSA [9], MS-275 [10], and SAHA [11] may induce differentiation, apoptosis and cell cycle arrest via the modulation of gene expression by histone acetylation. Van Lint et al. [12] demonstrated by differential display that the expression of 2% of genes is significantly changed in cultured cells after treatment with an HDAC inhibitor, TSA. Although HDAC inhibitors show antitumor effects by modulating gene expression, the precise profiles of gene expression related to HDAC inhibitors have yet to be elucidated. In this study, we detected genes sensitive to FK228 in vitro and identified marker genes to predict sensitivity to FK228 in vivo using Affymetrix GeneChip.

## 2. Materials and methods

### 2.1. Chemicals

FK228 was prepared at Fujisawa Pharmaceutical Co., Ltd. For in vitro studies, FK228 was dissolved in 100% ethanol and diluted with each experimental medium. For in vivo studies, FK228 was dissolved in and diluted with 10% polyoxyethylated hydrogenated castor oil in saline.

### 2.2. Tumor cells and animals

PC-3 (prostate cancer), ACHN and A-498 (renal cancer) were obtained from ATCC. U937 (histiocytic lymphoma) was obtained from the Institute of Development, Aging and Cancer, Tohoku University. SC-6 (stomach cancer) and LC-6 (lung large cell cancer) were obtained from the Central Institute for Experimental Animals. Lu-65 (lung large cell cancer) was obtained from the National Institute of Health Sciences. PC-3 and ACHN were maintained in Dulbecco's Modified Eagle's Medium (Nikken Biological Laboratories) supplemented with 10% fetal calf serum (Moregate), 50 units/mL of penicillin and 50 μg/mL of streptomycin (ICN Biomedicals). U937 was maintained in RPMI-1640 Medium (Nikken Biological Laboratories) supplemented with 10% fetal calf serum, 50 units/mL of penicillin and 50 μg/mL of streptomycin. Male nude mice were purchased from Charles River Japan Inc. PC-3, ACHN, A-498, SC-6, LC-6 and Lu-65 were maintained s.c. by serial passage in nude mice.

### 2.3. In vivo antitumor activity

Fragments (3 mm × 3 mm × 3 mm) of human solid tumor were implanted s.c. into the right flank of nude mice. When the estimated tumor weight in mice had reached 100–300 mg, animals were treated i.v. with 1.8 or 3.2 mg/kg of FK228 every 4 days for a total of three times. Six mice were used for each group. Tumor weight was calculated from the following formula: tumor weight (mg) = $(L \times W^2)/2$, where $L$ and $W$ represent the length and the width of the tumor mass, respectively. The final weight of A-498 tumor was measured 2 weeks after the first administration day, and the weights of other tumors were measured about 2 weeks after the first administration day as previously described [3,13]. The final tumor weights were used for the calculation of growth inhibition. Growth inhibition was calculated as follows: Growth inhibition (%) = ((1 − mean tumor weight of FK228 treated group/mean tumor weight of control group) × 100)

### 2.4. GeneChip analysis

In in vitro tests, cells were seeded at $4 \times 10^6$ per 75-$cm^2$ flask and incubated in each medium overnight at 37 °C under 5% $CO_2$. The following day, cells were incubated with 5 ng/mL of FK228, at 37 °C under 5% $CO_2$ for 1, 3, 12, and 24 h. RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN). In in vivo tests, fragments (3 mm × 3 mm × 3 mm) of human solid tumor were implanted s.c. into the right frank of nude mice. When the estimated tumor weight in mice had reached 100–300 mg, RNA was extracted from the solid tumors using RNeasy Midi Kit (QIAGEN). GeneChip analysis was based on the Affimetrix GeneChip Manual (Affimetrix). Total RNA was converted to double-strand cDNA using an oligo-dT primer containing the T7 RNA polymerase promoter (Superscript Choice System; Gibco-BRL). The cDNA was extracted with phenol/chloroform, ethanol precipitated, and converted to biotinylated cRNA using the Bioarray High Yield RNA Transcription Kit (Enzo Diagnostic). Biotinylated cRNA was cleaned and fragmented, and quality was confirmed by hybridizing a small portion to the Affimetrix Test2 Array. Ten micro grams of the biotinylated cRNA was hybridized to a GeneChip (Affimetrix FL array), and then the chip was washed and stained with streptavidin–phycoeryhrin. Chips were scanned with a probe array scanner, and data were analyzed with the Affimetrix Microarray Analysis Suite using human ß-actin, GAPDH, hexokinase, 5S rRNA, and B1/B2 repeats of gamma-crystalline as housekeepers genes; murine, rat and yeast probe sets as negative hybridization controls; and externally spiked bacterial bioB, bioC, bioD, and cre as positive hybridization controls. To determine the RNA level quantitatively, average differences representing perfectly matched minus mismatched for each gene-specific probe set were calculated. GeneSpring Software (SiliconGenetics) was used to examine differential gene expression. To detect truly expressed genes, row data for genes were filtered at more than 100.

### 2.5. Confirmation of gene expression patterns of MKP-1 and caspase 9 by RT-PCR

Fragments (3 mm × 3 mm × 3 mm) of human solid tumor were implanted s.c. into the right flank of nude mice. When the estimated tumor weight in mice had reached 100–300 mg, RNA was extracted from the solid tumors using RNeasy Midi Kit (QIAGEN) and reverse-transcribed using Taq-Man reverse transcription reagents (PE Biosystems). PCR was performed using recombinant Taq DNA polymerase (Takara Shuzo Co., Ltd.). Optimal reaction conditions for PCR were determined for each primer pair. PCR cycling parameters were denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, followed by elongation at 72 °C for 30 s. PCR products were analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. Primers for MKP-1, caspase 9 and ß-actin genes were designed using ''primer express'' software (PE Biosystems). The following primer sequences were used: 5′-GAC AAC CAC AAG GCA GAC ATC A-3′ (MKP-1 upstream), 5′-CAA ACA CCC TTC CTC CAG CAT-3′ (MKP-1 downstream); 5′-CAG GCA AGC AGC AAA GTT GTC-3′ (caspase 9 upstream), 5′-GAA CCT CTG GTT TGC GAA TCT C-3′ (caspase 9 downstream); 5′-TCA ACA CCC CAG CCA TGT ACG-3′ (ß-actin upstream), 5′-CAG GAA GGA AGG CTG GAA GAG-3′ (ß-actin downstream). The final expression value was calculated as follows: expression level of caspase 9 or MKP-1 mRNA/expression level of ß-action.

## 3. Results

### 3.1. Time-dependent alternation of gene expression by FK228 in human tumor cells in vitro

Genes time-dependently up- or down-regulated by FK228 in cultured cells were identified first, because the $p21^{WAF1/Cip1}$ gene, which is sensitive to FK228, has been shown to be time-dependently increased by FK228 [8,13]. FK228 inhibits the growth of various human tumor cell lines, such as U937 lymphoma, PC-3 prostate and ACHN renal cells, in vitro with $IC_{50}$ values of several ng/mL [8,13]. In this test, FK228 was used at a concentration of 5 ng/mL, based on the

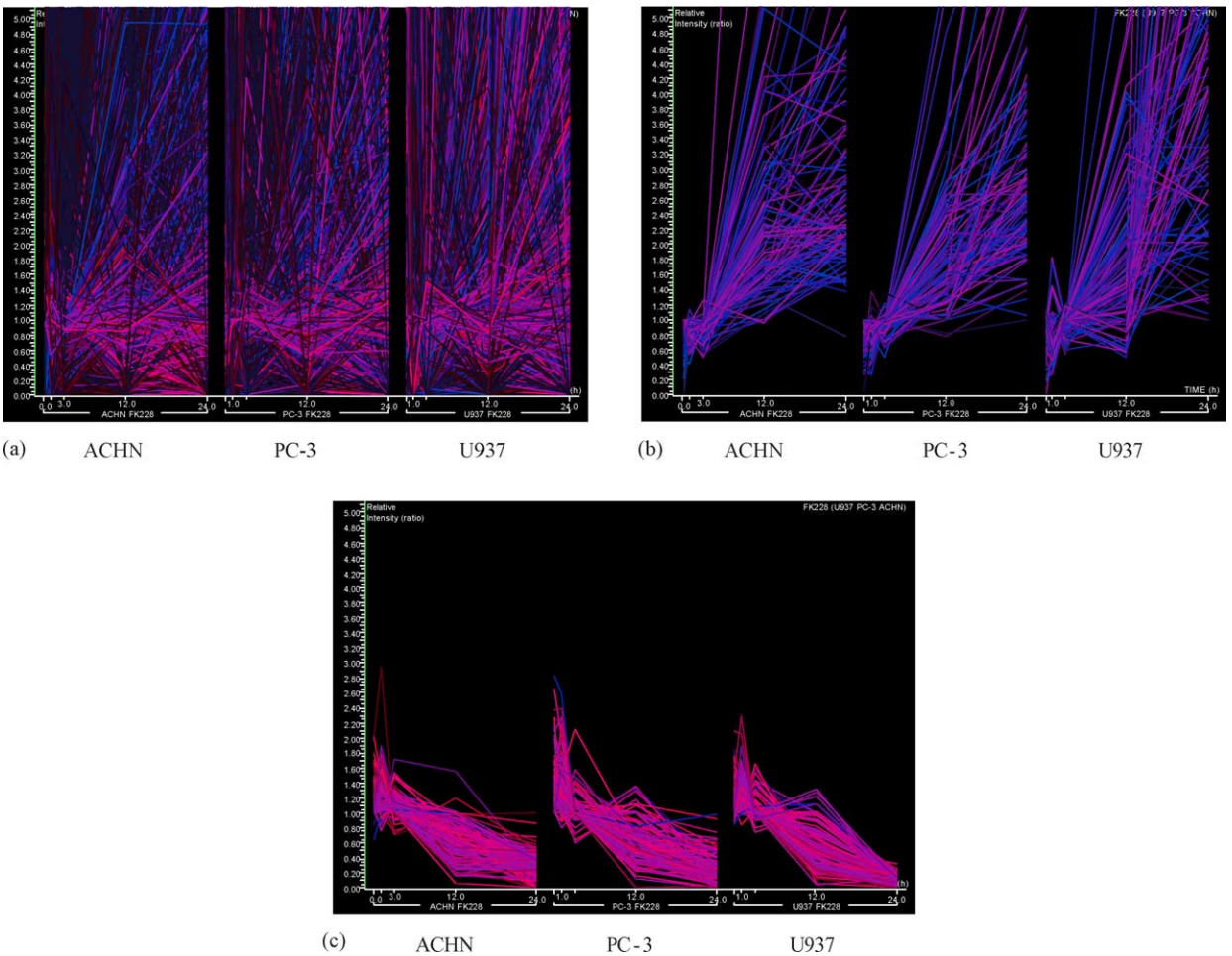


Fig. 1. Time-dependent alternation of gene expression in ACHN, PC-3, and U937 cells treated with FK228 in vitro. (a) All genes that changed expression in response to FK228; (b and c) genes time-dependently up- (b) or down- (c) regulated more than two-fold by FK228 at 24 h.

$IC_{50}$ values of the in vitro cytotoxicity tests [8,13]. Since 5 ng/mL of FK228 induced apoptosis of U937 cells at 20 h [8] and apoptosis of PC-3 at 36 h (data not shown), we used the treatment time of FK228 up to 24 h, which was near the time of the start of apoptosis. U937, PC-3 and ACHN cells were treated with 5 ng/mL of FK228 for 1, 3, 12, and 24 h, then RNA was isolated from cells to prepare target for probe array hybridization. Affimetrix FLGeneChip, which consists of the 7070 genes, was used for target hybridization and the data was analyzed using the following procedures with GeneSpring Software. First all genes that changed expression in response to FK228 were identified (Fig. 1a). Next, data were filtered to obtain genes that were time-dependently up- or down-regulated more than two-fold at 24 h by FK228 (Fig. 1b and c). Finally, genes which had the same change in the expression in all cell lines were selected. As shown in Tables 1 and 2, 3% of genes (205/7070) were commonly and time-dependently up- or down-regulated by FK228, 105 and 100 genes were up- and down-regulated, respectively, by FK228. Commonly up-regulated genes included $p21^{WAF1/Cip1}$, Interleukin-8 (IL-8), histone family, JunB, caspase 9, MKP-1, and MAPK family, and commonly down-regulated genes included cyclin A and MAPK family.

Table 1
List of genes up-regulated by FK228 in PC-3, ACHN and U937 in vitro

| List no. | Accession no. | Gene |
|---|---|---|
| 1 | U09579 | $p21^{WAF1/Cip1}$(mda-6) |
| 2 | U03397 | Tumor necrosis factor receptor superfamily, member 9 |
| 3 | M96843 | Id2B |
| 4 | V01512 | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| 5 | X51345 | JunB proto-oncogene |
| 6 | HG3105–HT3281 | |
| 7 | D17547 | Source: human mRNA for DOPAchrome tautomerase (tyrosinase-related protein-2), complete cds |
| 8 | M31516 | Decay accelerating factor for complement (CD55, Cromer blood group system) |
| 9 | M60974 | DNA-damage-inducible transcript 1 |
| 10 | U35048 | Transforming growth factor beta-stimulated protein TSC-22 |
| 11 | X60487 | H4/h |
| 12 | U65092 | Melanocyte specific gene 1 |
| 13 | L14848 | MHC class I polypeptide-related sequence A |
| 14 | U00115 | B-cell CLL/lymphoma 6 (zinc finger protein 51) |
| 15 | U60415 | |
| 16 | U40369 | Spermidine/spermine *N*1-acetyltransferase |
| 17 | HG2167–HT2237 | |
| 18 | X68277 | Mitogen-activated protein kinase phosphatase 1 (MKP-1) |
| 19 | L19872 | Aryl hydrocarbon receptor |
| 20 | D79206 | Syndecan 4 (amphiglycan, ryudocan) |
| 21 | U68111 | Source: human protein phosphatase inhibitor 2 (PPP1R2) gene, exon 6 and complete cds |
| 22 | X59405 | |
| 23 | D50645 | Stroma cell-derived factor-2 |
| 24 | Z29331 | Ubiquitin-conjugating enzyme E2H (homologous to yeast UBC8) |
| 25 | D26067 | Source: human mRNA for KIAA0033 gene, partial cds |
| 26 | U20734 | JunB |
| 27 | L19783 | Phosphatidylinositol glycan, class H |
| 28 | U12778 | Acyl-coenzyme A dehydrogenase, short/branched chain |
| 29 | U51269 | Armadillo repeat gene deletes in velocardiofacial syndrome |
| 30 | J04056 | Carbonyl reductase 1 |
| 31 | X75304 | Golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1 |
| 32 | D87440 | Similar to *Plasmodium falciparum* glutamic acid-rich protein precursor (A54514) (KIAA0252) |
| 33 | D50916 | Homolog of yeast (*Saccharomyces cerevisiae*) ufd2 |
| 34 | M31166 | Pentaxin-related gene, rapidly induced by interleukin-1 beta (PTX3) |
| 35 | X79353 | GDP dissociation inhibitor 1 |
| 36 | S73591 | 1,25-Dihydroxyvitamin D-3 up-regulated; This sequence comes from Fig. 2; protein sequence is in conflict with the conceptual translation; mismatch (26[K->R]) (brain-expressed HHCPA78 homolog) |
| 37 | X01703 | Source: human gene for alpha-tubulin (b alpha 1) |
| 38 | X85750 | Expression associated with monocyte to macrophage differentiation |
| 39 | S58733 | pp52 |
| 40 | D13435 | Phosphatidylinositol glycan, class F |
| 41 | U41654 | Putative GTP-binding protein, homolog of small GTPase family members (FIP-1) |
| 42 | X15673 | |
| 43 | U77735 | Similar to murine pim-2 product encoded by GenBank accession number L41495; serine/threonine protein kinase |
| 44 | U56998 | Cytokine-inducible kinase |
| 45 | L09209 | Amyloid beta (A4) precursor-like protein 2 |
| 46 | Y00787 | Interleukin-8 (IL-8) |

Table 1 (*Continued*)

| List no. | Accession no. | Gene |
|---|---|---|
| 47 | X61123 | B-cell translocation gene 1, anti-proliferative |
| 48 | M92843 | Zinc finger protein homologous to Zfp-36 in mouse |
| 49 | U60521 | Caspase 9, apoptosis-related cysteine protease |
| 50 | U60205 | Enzyme involved in the cholesterol biosynthetic pathway; iron is an essential cofactor needed for enzyme activity; human homologue of *Saccharomyces cerevisiae* C-4 methyl sterol oxidase (ERG25) |
| 51 | D85181 | |
| 52 | D64142 | H1 histone family, member X |
| 53 | X76717 | |
| 54 | X62078 | GM2 ganglioside activator protein |
| 55 | HG4668–HT5083 | |
| 56 | D87953 | Source: human mRNA for RTP, complete cds, (GC4) |
| 57 | U12387 | 35 kDa Monomer; cytosolic protein (TPMT) |
| 58 | J03077 | Prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy) |
| 59 | M24470 | Guanosine monophosphate reductase |
| 60 | M55998 | COL1A1 |
| 61 | M14949 | R-ras |
| 62 | X72964 | Caltractin (20 kDa calcium-binding protein) |
| 63 | U63825 | Isolated in a two hybrid screen to identify cellular proteins that interact with hepatitis delta antigen; similar to hepatitis delta antigen, and has two regions predicted to form coiled-coil protein interaction domains (dipA) |
| 64 | U03398 | Tumor necrosis factor (ligand) superfamily, member 9 |
| 65 | Y00451 | Aminolevulinate, delta-, synthase 2 (sideroblastic/hypochromic anemia) |
| 66 | X13916 | Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor) |
| 67 | D80010 | Source: human mRNA for KIAA0188 gene, partial cds |
| 68 | M37400 | Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1) |
| 69 | X56681 | JunD proto-oncogene |
| 70 | HG1828-HT1857 | |
| 71 | HG3543-HT3739 | |
| 72 | X78687 | Neuraminidase |
| 73 | X74331 | Primase, polypeptide 2A (58 kDa) |
| 74 | J03909 | Gamma-interferon-inducible protein precursor (IFI30) |
| 75 | M28130 | Source: human interleukin-8 (IL-8) gene, complete cds |
| 76 | U72515 | Similar to ESTs with GenBank accession numbers H45806 and H45837; similar to *Saccharomyces cerevisiae* ORF YOR175c encoded by GenBank accession number Z75083; see corresponding genomic sequence in GenBank accession number U72506 (C3f) |
| 77 | X17025 | Isopentenyl-diphosphate delta isomerase (IDI1) |
| 78 | U86070 | Phosphomannomutase 1 |
| 79 | M63379 | Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J) |
| 80 | L19779 | H2A histone family, member O |
| 81 | M76378 | Cysteine and glycine-rich protein 1 |
| 82 | L77567 | Source: *Homo sapiens* mitochondrial citrate transport protein (CTP) mRNA, 3′ end |
| 83 | D87116 | Mitogen-activated protein kinase kinase 3 (MKK3/MAPKK3) |
| 84 | M33764 | Ornithine decarboxylase 1 |
| 85 | U00968 | Sterol regulatory element binding transcription factor 1 |
| 86 | U22816 | LIP1b; serine phosphorylated; colocalizes with LAR at focal adhesions; LAR PTPase-associated; coiled-coil protein |
| 87 | M23575 | Pregnancy specific beta-1-glycoprotein 13 |
| 88 | U58516 | Breast epithelial antigen |
| 89 | X52151 | Arylsulfatase A |
| 90 | U46751 | Ubiquitin-binding protein P62; phosphotyrosine independent ligand for the Lck SH2 domain p62 |
| 91 | X71490 | Vacuolar proton-ATPase, subunit D; V-ATPase, subunit D (ATP6DV) |
| 92 | L38503 | Glutathione S-transferase theta 2 |
| 93 | X55448 | Source: *Homo sapiens* G6PD gene for glucose-6-phosphate dehydrogenase |
| 94 | U91316 | Source: human acyl-CoA thioester hydrolase mRNA, complete cds |
| 95 | X72755 | Monokine induced by gamma interferon |
| 96 | L13740 | Source: human TR3 orphan receptor mRNA, complete cds |
| 97 | M14764 | Nerve growth factor receptor (TNFR superfamily, member 16) |
| 98 | L09235 | ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha polypeptide, 70 kDa, isoform 1 (ATP6A1) |
| 99 | M60750 | H2B histone family, member L |
| 100 | M31724 | Protein tyrosine phosphatase, non-receptor type 1 |
| 101 | X69878 | fms-Related tyrosine kinase 4 |
| 102 | M37457 | Source: human $Na^+$-, $K^+$-ATPase catalytic subunit alpha-III isoform gene, exon 23, clone lambda-NK-alpha-R3-2; (ATP1A3) |
| 103 | S56151 | Milk fat globule protein; This sequence comes from Fig. 3 (HMFG) |
| 104 | U79288 | |
| 105 | Z68228 | Junction plakoglobin |

Table 2
List of genes down-regulated by FK228 in PC-3, ACHN and U937 in vitro

| List no. | Accession no. | Gene |
|---|---|---|
| 1 | U07620 | Jun-N-terminal kinase 3/mitogen-activated protein kinase 10 (JNK3/MAPK10) |
| 2 | D00591 | Chromosome condensation 1 |
| 3 | M21188 | Insulin-degrading enzyme |
| 4 | M34458 | lamin B |
| 5 | U10324 | Interleukin enhancer binding factor 3, 90 kDa |
| 6 | U21551 | Source: human ECA39 mRNA, complete cds |
| 7 | U15637 | TNF receptor-associated factor 3 |
| 8 | X78992 | Source: *Homo sapiens* ERF-2 mRNA |
| 9 | D50922 | KIAA0132 gene product |
| 10 | Z69915 | |
| 11 | D00596 | Thymidylate synthetase |
| 12 | M80647 | Thromboxane A synthase 1 (platelet, cytochrome P450, subfamily V) |
| 13 | X14008 | Protein sequence is in conflict with the conceptual translation |
| 14 | X89416 | Source: *Homo sapiens* mRNA for protein phosphatase 5 |
| 15 | L23959 | Source: *Homo sapiens* E2F-related transcription factor (DP-1) mRNA, complete cds |
| 16 | Y10375 | SIRP-alpha1 |
| 17 | U77604 | Microsomal glutathione S-transferase 2 |
| 18 | L39874 | dCMP deaminase (DCTD) |
| 19 | M94362 | Source: human lamin B2 (LAMB2) mRNA, partial cds |
| 20 | D85131 | Source: *Homo sapiens* mRNA for Myc-associated zinc-finger protein of human islet, complete cds |
| 21 | X71874 | Proteasome (prosome, macropain) subunit, beta type, 10 |
| 22 | J03801 | Lysozyme (renal amyloidosis) |
| 23 | D83783 | Thyroid hormone receptor-associated protein, 230 kDa subunit |
| 24 | X95808 | X-linked mental retardation candidate gene (DXS6673E) |
| 25 | U09578 | Mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK-3) |
| 26 | U08815 | Similar to yeast PRP9, Swiss-Prot accession number P19736 (SAP 61) |
| 27 | D55716 | Source: human mRNA for P1cdc47, complete cds |
| 28 | L76191 | Interleukin-1 receptor-associated kinase 1 |
| 29 | U18237 | Similar to the product encoded by GenBank accession number Z19209 |
| 30 | X62048 | Wee1+ (*S. pombe*) homolog |
| 31 | J04088 | Topoisomerase (DNA) II alpha (170 kDa) |
| 32 | AC002450 | Similar to ADP/ATP carrier proteins; match to EST AA360112 (NID: g2012453), similar to U00052 (PID:g485131) (WUGSC:H_GS244B22.1) |
| 33 | U09087 | Thymopoietin |
| 34 | U18271 | Source: human thymopoietin (TMPO) gene, partial exon 6, complete exon 7, partial exon 8, and partial cds for thymopoietin beta |
| 35 | D78335 | Source: human mRNA for 5′-terminal region of UMK, complete cds |
| 36 | U70439 | Similar to human PHAPI2a encoded by GenBank accession number Y07569 and human PHAPI2b encoded by GenBank accession number Y07570 |
| 37 | D84557 | Source: *Homo sapiens* mRNA for HsMcm6, complete cds |
| 38 | Z14982 | Alternative splicing (MHC-encoded proteasome subunit gene LAMP7-E1) |
| 39 | U09937 | Source: human urokinase-type plasminogen receptor, exon 7 |
| 40 | D13645 | KIAA0020 gene product |
| 41 | X62153 | |
| 42 | U32680 | Ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease) |
| 43 | D21063 | Minichromosome maintenance deficient (*Saccharomyces cerevisiae*) 2 (mitotin) |
| 44 | M98045 | Folylpolyglutamate synthase |
| 45 | X68688 | ZNF33B |
| 46 | X65550 | Antigen identified by monoclonal antibody Ki-67 |
| 47 | D38076 | RAN binding protein 1 |
| 48 | X74795 | Source: *Homo sapiens* P1-Cdc46 mRNA |
| 49 | D38073 | Minichromosome maintenance deficient (*Saccharomyces cerevisiae*) 3 |
| 50 | J03589 | Ubiquitin-like protein (UBL4) |
| 51 | L05147 | Dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related) |
| 52 | U07802 | Source: human Tis11d gene, complete cds |
| 53 | L20010 | |
| 54 | U58090 | Cullin 4A |
| 55 | U14518 | Centromere protein A (17 kDa) |
| 56 | U63743 | HsMCAK |
| 57 | Z74792 | Source: *Homo sapiens* mRNA for CCAAT transcription binding factor subunit gamma |
| 58 | U31930 | dUTP pyrophosphatase |
| 59 | U50939 | Amyloid beta precursor protein-binding protein 1, 59 kDa |
| 60 | D50678 | Low density lipoprotein receptor-related protein 8 |
| 61 | U80184 | Flightless I (Drosophila) homolog (FLII) |

Table 2 (*Continued*)

| List no. | Accession no. | Gene |
|---|---|---|
| 62 | X74794 | Minichromosome maintenance deficient (*Saccharomyces cerevisiae*) 4 |
| 63 | D85425 | CAATT box binding factor subuit C |
| 64 | X51688 | Cyclin A2 |
| 65 | U18271 | Source: human thymopoietin (TMPO) gene, partial exon 6, complete exon 7, partial exon 8, and partial cds for thymopoietin beta |
| 66 | U58087 | Cullin 1 |
| 67 | U05875 | Second chain of the receptor (IFNGR2) |
| 68 | D32002 | Nuclear cap binding protein, 80 kDa |
| 69 | U52101 | Epithelial membrane protein 3 |
| 70 | U93867 | Source: human RNA polymerase III subunit (RPC62) mRNA, complete cds |
| 71 | U06681 | |
| 72 | M83233 | Transcription factor 12 (HTF4, helix-loop-helix transcription factors 4) |
| 73 | U73477 | LANP; PHAPI; I-1pp2a |
| 74 | D50919 | KIAA0129 gene product |
| 75 | U83115 | Absent in melanoma 1 |
| 76 | X63692 | DNA (cytosine-5-)-methyltransferase 1 |
| 77 | M31627 | X-box binding protein 1 |
| 78 | D26018 | Source: human mRNA for KIAA0039 gene, partial cds |
| 79 | L10838 | Splicing factor, arginine/serine-rich 3 |
| 80 | D87448 | Similar to *S. pombe*-rad4+/cut5+ roduct (A40727) (KIAA0259) |
| 81 | U40152 | Origin recognition complex, subunit 1 (yeast homolog)-like |
| 82 | D87673 | Heat shock transcription factor 4 |
| 83 | D31889 | Source: human mRNA for KIAA0072 gene, partial cds |
| 84 | X62534 | High-mobility group (nonhistone chromosomal) protein 2 |
| 85 | Y13115 | Source: *Homo sapiens* mRNA for serine/threonine protein kinase SAK |
| 86 | U10886 | Transmembrane protein |
| 87 | U32849 | N-myc (and STAT) interactor |
| 88 | L13800 | |
| 89 | X74987 | Ribonuclease L (2′,5′-oligoisoadenylate synthetase-dependent) inhibitor |
| 90 | U13045 | GA-binding protein transcription factor, beta subunit 2 (47 kDa) |
| 91 | Z23064 | Heterogeneous nuclear ribonucleoprotein G |
| 92 | X67155 | Mitotic kinesin-like protein 1 |
| 93 | X85137 | |
| 94 | X86098 | Binds directly to adenovirus type 5 E1A protein (BS69) |
| 95 | L40384 | Thyroid receptor interacting protein 13 |
| 96 | D25538 | Adenylate cyclase 7 |
| 97 | X66113 | Polymyositis/scleroderma autoantigen 2 (100 kDa) |
| 98 | X59739 | Zinc finger protein, X-linked |
| 99 | X96586 | Neutral sphingomyelinase (N-SMase) activation associated factor |
| 100 | L78833 | Similar to ribosomal protein L21 (BRCA1) |

### 3.2. Native gene expression profiles of human tumor xenografts

Next, marker genes to predict sensitivity to FK228 were examined in vivo. FK228 showed antitumor activity with differential sensitivity against various tumors implanted in mice, although it inhibited the growth of various tumor cell lines to the same degree in vitro [3,13]. Genes with native differences in patterns of expression between FK228-sensitive and resistant tumors implanted in nude mice were identified. PC-3 prostate and SC-6 stomach xenografts were used as FK228-sensitive tumors, while ACHN and A-498 renal xenografts were used as FK228-resistant tumors (Table 3). Data was analyzed according to the following procedures. First, hierarchical clustering analysis was performed and genes were classified into a phylogenetic tree (Fig. 2a). In the tree, similar kinds of genes were clustered together. Next, genes which had at least two-fold differences in expression between sensitive and resistant tumors were identified, based on statistical information provided by Affymetrix (Fig. 2b–e). Finally, 1% of genes (76/7070) were identified by native differences in the patterns of expression between FK228-sensitive and resistant tumors. Twenty-seven and forty nine of those genes were expressed at high or low levels, respectively, in FK228-sensitive tumors (Tables 4 and 5).

Table 3
Antitumor activity of FK228 against human tumor xenografts

| Sensitivity to FK228 | Tumor | Growth inhibition (%) | | References |
|---|---|---|---|---|
| | | 1.8 mg/kg | 3.2 mg/kg | |
| Sensitive | PC-3 | 87 | 98 | [13] |
| | SC-6 | 77 | 84 | [3] |
| Resistant | ACHN | 17 | 20 | [13] |
| | A-498 | 31 | 29 | This study |

Tumor cells were inoculated s.c. into nude mice. Drugs were given i.v. to mice three times at 4-day intervals. Six mice were used for each group. The final tumor weights were measured about 2 weeks after the first administration day and used for the calculation of growth inhibition.

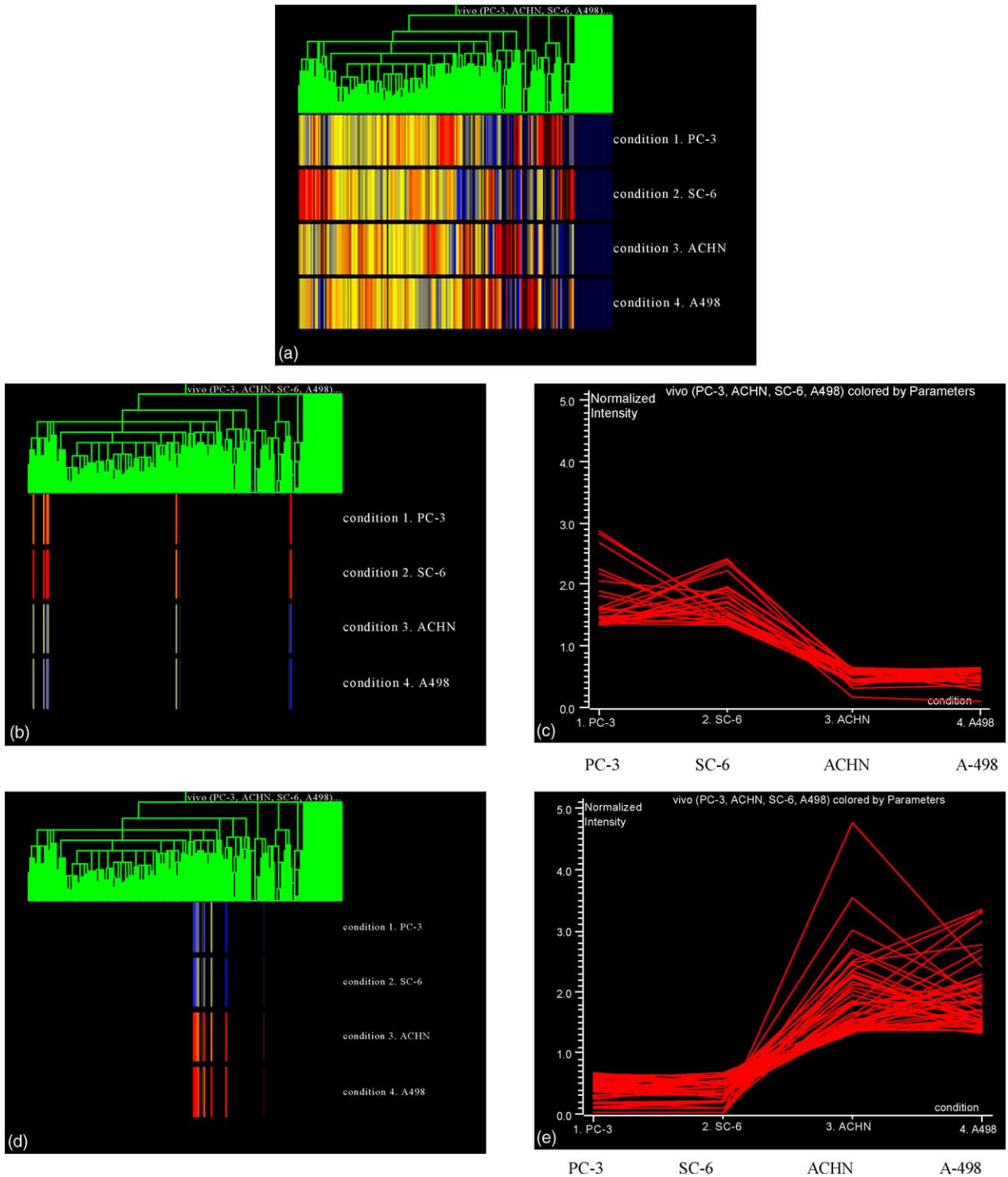


Fig. 2. Hierarchical cluster analysis of gene expression profiles in PC-3, SC-6, ACHN, and A-498 tumors. (a) Genes classified into a phylogenetic tree comparing PC-3, SC-6, ACHN, and A-498 tumors, blue color is indicative of decreased expression and red color indicates an increased expression; (b and c) genes with high expression in FK228-sensitive tumors or low expression in FK228-resistant tumors; (d and e) genes with low expression in FK228-sensitive tumors or high expression in FK228-resistant tumors.

### *3.3. Expression patterns of caspase 9 and MKP-1 in human tumor xenografts*

Caspase 9 and MKP-1 genes showed distinct differences in patterns of expression between FK228-sensitive and resistant tumors (Fig. 3). Expression of the caspase 9 gene in FK228-sensitive tumors was higher than that in FK228-resistant tumors (expression level of caspase 9 in FK228-sensitive versus resistant tumors, $134.0 \pm 41.1$ versus $27.2 \pm 1.7$, respectively) and expression of the MKP-1

Table 4
List of the genes with high expression in FK228-sensitive tumor and low expression in FK228-resistant tumor in vivo

| List no. | Accession no. | Gene |
|---|---|---|
| 1 | L11005 | Aldehyde oxidase 1 |
| 2 | X12517 | Small nuclear ribonucleoprotein polypeptide C |
| 3 | U43944 | Malic enzyme 1, soluble |
| 4 | U52100 | Epithelial membrane protein 2 |
| 5 | U65579 | NADH dehydrogenase (ubiquinone) Fe-S protein 8 (23 kDa) (NADH-coenzyme Q reductase) |
| 6 | X06272 | Signal recognition particle receptor ('ocking protein') |
| 7 | D42047 | The ha3662 gene product is related to mouse glycerophosphate dehydrogenase |
| 8 | D90086 | Pyruvate dehydrogenase (lipoamide) beta |
| 9 | U61734 | |
| 10 | J03798 | Small nuclear riboprotein Sm-D |
| 11 | X05299 | Centromere protein B (80 kDa) |
| 12 | L34155 | Laminin, alpha 3 (nicein (150 kDa), kalinin (165 kDa), BM600 (150 kDa), epilegrin) |
| 13 | U60521 | Caspase 9, apoptosis-related cysteine protease |
| 14 | Y10807 | HMT1 (hnRNP methyltransferase, *Saccharomyces cerevisiae*)-like 2 |
| 15 | U44754 | Small nuclear RNA activating complex, polypeptide 1, 43 kDa |
| 16 | U78107 | *N*-ethylmaleimide-sensitive factor attachment protein, gamma |
| 17 | U60644 | Similar to Vaccinia virus *Hin*dIII K4L ORF, and to Vaccinia virus p37 (*Hin*dIII F13L ORF) |
| 18 | U32986 | Damage-specific DNA binding protein 1 (127 kDa) |
| 19 | D59253 | RNA-binding protein that has two RNP consensus motifs |
| 20 | U41767 | A disintegrin and metalloproteinase domain 15 (metargidin) |
| 21 | U10550 | Source: human Gem GTPase (gem) mRNA, complete cds |
| 22 | D38521 | The ha0919 gene product is novel |
| 23 | J04823 | Cytochrome *c* oxidase subunit VIII |
| 24 | M21154 | S-adenosylmethionine decarboxylase 1 |
| 25 | X78565 | Hexabrachion (tenascin C, cytotactin) |
| 26 | D50405 | histone deacetylase 1 |
| 27 | X04366 | Calpain, large polypeptide L1 |

gene in FK228-resistant tumors was higher than that in FK228-sensitive tumors ($P < 0.01$ for expression level of MKP-1 in FK228-sensitive versus resistant tumors, $351.3 \pm 39.2$ versus $882.2 \pm 3.1$, respectively). The difference in expression level of caspase 9 between FK228-sensitive and resistant tumors was not statistically significant but was more than three-fold, because the expression level of caspase 9 in PC-3 was higher than that in SC-6 (expression levels of caspase 9 in PC-3 and SC-6 were 175.1 and 92.9, respectively). To verify the patterns of expression of caspase 9 and MKP-1 genes indicated by GeneChip analysis, we examined the expression of those genes in FK228-sensitive or resistant tumors using RT-PCR. The expression of caspase 9 gene was higher in FK228-sensitive tumors (PC-3 and SC-6) ($P < 0.05$ for expression level ofcaspase 9 in FK228-sensitive versus resistant tumors, $1.16 \pm 0.07$ versus $0.74 \pm 0.03$, respectively) and the expression of MKP-1 gene was higher in FK228-resistant tumors (ACHN and A-498) ($P < 0.05$ for expression level of MKP-1 in FK228-sensitive versus resistant tumors, $1.02 \pm 0.02$ versus $1.87 \pm 0.13$, respectively) (Fig. 4a–c). Although the expression levels of those genes in FK228-sensitive and resistant tumors were different between GeneChip and RT-PCR analysis, the expression patterns of those genes in FK228-sensitive and resistant tumors by RT-PCR analysis were similar to those by GeneChip analysis. To investigate whether caspase 9 and MKP-1 genes may be used as marker genes to predict sensitivity to FK228, patterns of expression of those genes in other xenografts were examined. Lu65 and LC-6 xenografts are FK228-sensitive tumors (Table 6). Patterns of expression of caspase 9 and MKP-1 genes in those xenografts were the same as in FK228-sensitive PC-3 and SC-6 xenografts (Fig. 4a). From the result of 6 tumors, the expression of caspase 9 gene was higher in FK228-sensitive tumors ($P < 0.05$ for expression level of caspase 9 in FK228-sensitive versus resistant tumors, $1.10 \pm 0.05$ versus $0.74 \pm 0.03$, respectively) and expression of MKP-1 gene was higher in FK228-resistant tumors ($P < 0.05$ for expression level of MKP-1 in FK228-sensitive versus resistant tumors, $0.62 \pm 0.25$ versus $1.87 \pm 0.13$, respectively) (Fig. 4d and e).

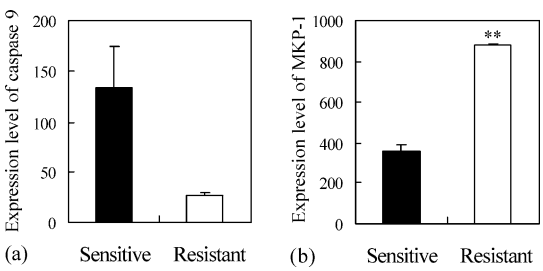


Fig. 3. Expression levels of caspase 9 (a) and MKP-1 (b) genes in FK228-sensitive (PC-3 and SC-6) and resistant (ACHN and A-498) tumors by GeneChip analysis. Results are shown as means ± S.E.M. and analyzed for statistical significance by unpaired *t*-test ($^{**}P < 0.01$).

Table 5
List of the genes with low expression in FK228-sensitive tumor and high expression in FK228-resistant tumor in vivo

| List no. | Accession no. | Gene |
|---|---|---|
| 1 | U84487 | Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin) |
| 2 | M33600 | Major histocompatibility complex, class II, DR beta 5 |
| 3 | L24564 | Ras-related associated with diabetes |
| 4 | J03474 | Amyloid A precursor |
| 5 | J05428 | UDP glycosyltransferase 2 family, polypeptide B7 |
| 6 | L03840 | Fibroblast growth factor receptor 4 |
| 7 | M35878 | Insulin-like growth factor binding protein 3 |
| 8 | HG4318–HT4588 | |
| 9 | X87241 | FAT tumor suppressor (drosophila) homolog |
| 10 | M59807 | Natural killer cell transcript 4 |
| 11 | HG2379–HT3996 | |
| 12 | U37546 | Apoptosis inhibitor 1 |
| 13 | X72889 | Source: *Homo sapiens* hbrm mRNA |
| 14 | U66838 | Cyclin A1 |
| 15 | Z23090 | Heat shock 27 kDa protein 1 |
| 16 | U16031 | Signal transducer and activator of transcription 6, interleukin-4 induced |
| 17 | D84110 | Alternative splicing (see also D84107–D84111) |
| 18 | M55998 | COL1A1 |
| 19 | D28124 | Neuroblastoma candidate region, suppression of tumorigenicity 1 |
| 20 | HG3548–HT3749 | |
| 21 | X86809 | Phosphoprotein enriched in astrocytes 15 |
| 22 | M79462 | Promyelocytic leukaemia |
| 23 | M59465 | Tumor necrosis factor, alpha-induced protein 1 (endothelial) |
| 24 | HG2379–HT3997 | |
| 25 | Z36715 | ELK3, ETS-domain protein (SRF accessory protein 2) note: symbol and name provisional |
| 26 | U41654 | Putative GTP-binding protein, homolog of small GTPase family members |
| 27 | U72882 | Source: human interferon-induced leucine zipper protein (IFP35) mRNA, partial cds |
| 28 | U72649 | Rat PC3 and murine TIS21 genes homolog |
| 29 | U47621 | |
| 30 | L22214 | Adenosine A1 receptor |
| 31 | U68494 | Source: human hbc647 mRNA sequence |
| 32 | X69699 | Paired box gene 8 |
| 33 | X63717 | Tumor necrosis factor receptor superfamily, member 6 |
| 34 | U04285 | |
| 35 | L38969 | Source: *Homo sapiens* thrombospondin 3 (THBS3) mRNA, complete cds |
| 36 | Y10032 | Source: *Homo sapiens* mRNA for putative serine/threonine protein kinase |
| 37 | L48513 | Paraoxonase 2 |
| 38 | U89942 | Lysyl oxidase-like 2 |
| 39 | Z12173 | Glucosamine (*N*-acetyl)-6-sulfatase (Sanfilippo disease IIID) |
| 40 | U59423 | Sma and Mad homolog |
| 41 | X68277 | Mitogen-activated protein kinase phosphatase 1 (MKP-1) |
| 42 | U45878 | HIAP-1 |
| 43 | L08187 | |
| 44 | X71874 | Proteasome (prosome, macropain) subunit, beta type, 10 |
| 45 | D50863 | Testis-specific kinase 1 |
| 46 | X91911 | |
| 47 | U65011 | Encodes tumor antigen recognized by cytolytic T lymphocytes |

Table 6
Antitumor activity of FK228 against human tumor xenografts

| Tumor | Growth inhibition (%) | | References |
|---|---|---|---|
| | 1.8 mg/kg | 3.2 mg/kg | |
| Lu65 | 63 | 74 | [3] |
| LC-6 | 64 | 74 | [3] |

Tumor cells were inoculated s.c. into nude mice. Drugs were given i.v. to mice three times at 4-day intervals. Six mice were used for each group. The final tumor weights were measured about 2 weeks after the first administration day and used for the calculation of growth inhibition.

## 4. Discussion

Although HDAC inhibitors such as FK228 [8], TSA [9], MS-275 [10] and SAHA [11] show antitumor effects by modulating the expression of target genes, the precise profiles of gene expression related to HDAC inhibitors have yet to be elucidated. In this study, we detected genes sensitive to FK228 in vitro and identified marker genes to predict sensitivity to FK228 in vivo using Affymetrix GeneChip.

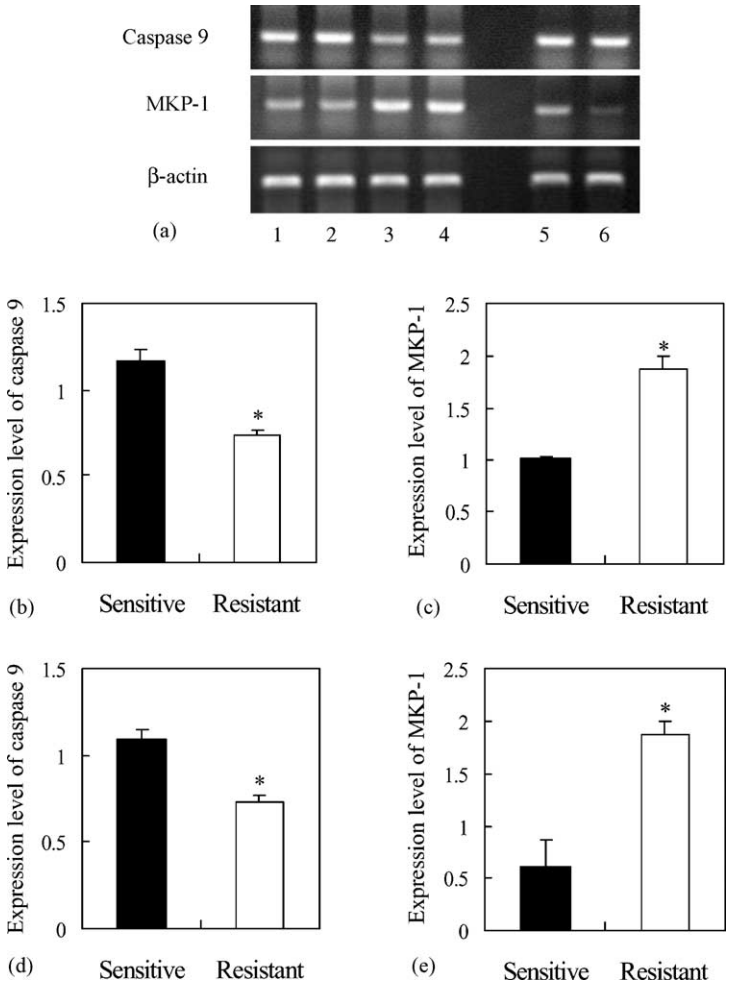


Fig. 4. Expression levels of caspase 9 and MKP-1 genes in FK228-sensitive and resistant tumors by RT-PCR analysis. (a) PC-3 (lane 1), SC-6 (lane 2), ACHN (lane 3), A-498 (lane 4), Lu65 (lane 5), and LC-6 (lane 6); (b and c) PC-3 and SC-6 were used as FK228-ssensitive tumors and ACHN and A-498 were used as FK228-resistant tumors; (d and e) PC-3, SC-6, Lu65 and LC-6 were used as FK228-sensitive tumors and ACHN and A-498 were used as FK228-resistant tumors. Results are shown as means ± S.E.M. and analyzed for statistical significance by unpaired *t*-test ($^{*}P < 0.05$).

First, genes sensitive to FK228 were identified using three cell lines in vitro. Very strict criteria were set in order to remove noise and select genes commonly and time-dependently up- and down-regulated by FK228 by more than two-fold. In this study, 3% of genes (205/7070) were affected by FK228 (Tables 1 and 2). Van Lint et al. [12] demonstrated by differential display that the expression of 2% of genes significantly changed in cultured cells after treatment with an HDAC inhibitor, TSA, which broadly agrees with the figures obtained in this study. These results suggest that HDAC inhibitors constantly modulate 2–3% of genes. Some of the 205 genes affected by FK228, such as $p21^{WAF1/Cip1}$, IL-8 and cyclin A, have been shown to be modulated by the other HDAC inhibitors [14–19]. FK228 time-dependently induced the expression of $p21^{WAF1/Cip1}$ and it has been reported that TSA, sodium butyrate and MS-275 induce the expression of $p21^{WAF1/Cip1}$ [14–16]. FK228 also increased the expression of IL-8 and TSA-induced hyperacetylation of the NF-κB-dependent receptor on IL-8 promoter has been reported, resulting in increased IL-8 mRNA transcription [17]. As similar to FK228, trapoxin and MS-275 down-regulating expression of cyclin A in human cancer cells [18,19]. Those studies of the other HDAC inhibitors support the reliability of data obtained in this study. Interestingly, four genes modulated by FK228 were associated with histone and all of them were up-regulated by FK228 (Table 1). It has been reported that the expression of H1 were induced by sodium butyrate and TSA in peripheral blood lymphocytes and this induced expression was found to be correlated with the concomitant induction of apoptosis and increased levels of histone H4 acetylation [20]. Histone acetylation is known to be involved in chromatin remodeling events [20]. These results indicated that the expression of histone family genes is increased via the histone acetylation by HDAC inhibitors and it may play roles in apoptosis and chromatin remodeling.

Expression of JunB is also induced by FK228. JunB is a component of the Jun family genes of the activating protein-1 transcription factors that are important in the control of cell growth and differentiation and neoplastic transformation [21]. Recently, it was demonstrated that

transgenic mice specifically lacking JunB expression in the myeloid lineage developed a myeloproliferative disease, which resembled human CML [21]. Expression levels of JunB were significantly impaired in the peripheral blood of CML patients and down-regulated JunB expression in CML was due to the inactivation of JunB gene by methylation [21]. Demethylation by treatment of human leukemia cells with 5′-aza-2′-deoxycytidine resulted in partial reactivation of JunB expression [21]. HDAC inhibitors treatment in combination with demethylation agents may show therapeutic efficacy on CML by inducing the expression of JunB gene.

The genes of MAPK family were modulated by FK228. MKK3 gene was increased and JNK3 and MAPKAPK-3 genes were decreased by FK228. MKK3 is the immediate upstream activator of p38 [22]. The effect of HDAC inhibitors on MKK3 has not been known, but HD AC inhibitors, SAHA and sodium butyrate activated p38 MAPK and induced apoptosis in human leukemia cells [23]. HDAC inhibitors may activate p38 MAPK by up-regulation of MKK3.

JNK3 is expressed predominantly in the nervous system and *Jnk3*$^{-/-}$ mice are resistant to exitotoxic killing of neurons [24]. MAPKAPK-3 is a substrate of p38 MAPK, which is activated in response to stress and cytokines [25]. The down-regulation of those genes by FK228 seems to be contradictory to FK228-inducted apoptosis in cultured cells [8]. Treatment with TSA attenuated the activations of p38 and ERK kinases by nocodazole and increased the chromosomal instability in HeLa cells [26]. Under such a condition, down-regulation of those genes by HDAC inhibitors may contribute to the chromosomal instability.

Next, marker genes to predict sensitivity to FK228 were identified in vivo. Using human tumors implanted in nude mice, in order to obtain the genes relevant to chemosensitivity has advantages. Accumulated evidence suggests that chemosensitivities in xenografts tend to agree with clinical results [27,28]. There are not much FK228-resistant human tumors transplantable to nude mice [3,13]. PC-3 and SC-6 were used as FK228-sensitive tumors, while ACHN and A-498 were used as FK228-resistant tumors. We identified 1% of genes (76/7070) were native differences in patterns of expression between FK228-sensitive and FK228-resistant tumors (Tables 4 and 5). In those genes, caspase 9 and MKP-1 genes were distinctly different in their patterns of expression, according to their sensitivity to FK228.

Expression of caspase 9 gene was natively high in FK228-sensitive tumors (Fig. 3) and induced by FK228 in cultured cells (Table 1). Caspase 9 is a member of the caspase family of cysteine proteases that are implicated in apoptosis [29]. Transduction of caspase 9 enhanced radiation-induced apoptosis [30] and activation of caspase 9 is required for UV- and cyclophosphamide-induced apoptosis [31,32]. *Caspase-9*$^{-/-}$ embryonic stem cells, embryonic fibroblasts and thymocytes are resistant to gamma-irradiation-induced apoptosis [33]. Although caspase 3 contributes to the apoptosis induced by HDAC inhibitors, HDAC inhibitors can induce the apoptosis in MCF-7, a cell line that does not express caspase 3 activity [34]. In MCF-7 cells, caspase 9 is critical for apoptosis induced by SAHA and TSA and proteolytic activation of caspase 2, caspase 8 and caspase 7 depends on caspase 9 [34]. Taken together these results suggest, natively high or induced expression of caspase 9 gene may relate to sensitivity to FK228 and play a role in FK228-induced apoptosis.

Expression of MKP-1 gene was natively low in FK228-sensitive tumors (Fig. 3) and induced by FK228 in cultured cells (Table 1). MKP-1 is a subclass of the protein tyrosine phosphatase gene superfamily and inactivates MAPKs by dephosphorylation of phosphothreonine and phosphotyrosine residues [35]. MKP-1 was originally identified as an ERK-specific phosphatase [36]. It is now well established that MKP-1 is also capable of inactivating both JNK and p38 [35]. Treatment with TSA attenuated the activation of p38 and ERK kinases by nocodazole and induced the chromatin instability in HeLa cells [26]. TSA also increased MKP-1 expression in C3H 10T1/2 cells [37]. Inactivation of p38 and ERK kinases may be due to the induction of MKP-1 by HDAC inhibitors. It has been reported that expression of MKP-1 is induced by a variety of extracellular stimuli and phosphorylation-acetylation of histone H3 precedes the induction of MKP-1 mRNA [37]. These results indicate that chromatin remodeling after stress contributes to the transcription induction of MKP-1 [37]. Induction of MKP-1 gene by FK228 may indicate that the chromatin remodeling is progressing.

Profiles of gene expression of human tumors implanted in nude mice after treatment with FK228 using GeneChip were also examined (data not shown). Although FK228 modulated expression of genes, such as $p21^{WAF1/Cip1}$ gene, the change in expression was not more than two-fold compared to before treatment with FK228. In GeneChip analysis, a change of less than two-fold in the expression of a gene is too small to distinguish true positives from false positives. Previously, we reported that FK228 induced expression of $p21^{WAF1/Cip1}$ gene more than two-fold in PC-3 xenograft after treatment with FK228 using real-time quantitive-PCR [13]. Taken together these results suggest that, although FK228 modulates not only the expression of target genes in vitro but also in vivo, modulation levels of genes in vivo may be smaller than those in vitro. Real-time quantitive-PCR analysis may be more sensitive for detecting the small changes in expression of genes than GeneChip analysis.

Recent developments of GeneChip and cDNA microarray technology have made it possible to analyze expression of thousands of genes at once [38–41]. Consequently, new classifications of cancer types can be proposed on the basis of altered expression of multiple genes in tumor tissue [42,43]. Scherf et al. [44] reported that profiles of gene expression might reflect drug sensitivity of cancer cells. Our results present profiles of gene expression related

to FK228 and marker genes to predict sensitivity to FK228, such as caspase 9 and MKP-1 genes, using Affymetrix GeneChip.

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