

Molecular features linked to the growth-inhibitory effects of gemcitabine on human pancreatic cancer cells

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Abstract. Although gemcitabine (GEM) is widely used in the treatment of pancreatic cancers, the molecular mechanisms that underlie its anti-tumor effects are not fully understood. To clarify the anti-tumor mechanism(s) of GEM, we studied a human pancreatic cancer cell line, YPK-1, that showed a 50% inhibitory concentration (IC₅₀) of GEM of 6.3×10^{-3} μ g/ml after 72 h of exposure. Cell proliferation was perturbed by 6 to 72 h of exposure to GEM concentrations equal to one-half or one-quarter of the IC₅₀. We used cDNA microarrays containing 2976 genes to identify genes with expression affected by exposure to GEM. The self-organizing map identified nine clusters, including 85 and 87 genes, that showed differential expression in response to exposure to one half and one quarter IC₅₀ GEM, respectively. Of these, 24 genes were common to cells exposed to the two different concentrations of GEM. Most are signal transduction or transcription-related genes. The microarray data for two of these genes, SPARC and RPS8, were validated by RT-PCR. Although further studies are needed to examine whether the changes in expression profiles of these genes are specific to cells exposed to GEM, the present data provide insights into the anti-tumor effects of GEM on pancreatic cancers.

Introduction

Pancreatic cancer is a devastating disease with a short survival after diagnosis because of its high metastatic potential (1,2). The development of anti-cancer drugs is necessary for the effective treatment of pancreatic cancer. Gemcitabine (GEM) is a novel nucleoside analogue that has significant anti-tumor activity against various solid tumors, including pancreatic

cancer, head and neck cancer, and non-small cell lung cancer (3-5), and it is more effective than fluorouracil in reducing symptoms in pancreatic cancer patients. Because GEM confers a small survival benefit to patients with pancreatic cancer, it is used as a first-line therapy for patients with advanced pancreatic cancer (6).

GEM does not always induce a complete response in all types of tumors; however, many studies have shown its clinical efficacy in the treatment of pancreatic cancer (7,8). GEM is phosphorylated and metabolized and then incorporated into cellular DNA. As a result, it causes masked chain termination (9,10). GEM sensitivity of cells is known to be affected by pathways that involve ribonucleotide reductase E2F1 and Bcl-2 (11-13). However, these pathways cannot account for all of the biological effects of GEM on cells, and therefore other pathways should be investigated. To clarify the mechanisms by which cell proliferation is perturbed by GEM, it is important to identify genes that show differential expression in response to GEM.

The development of cDNA microarray technology has allowed innovative studies of the levels of expression of thousands of genes with a single experiment. In the present study, we used cDNA microarray technology to investigate the mechanism that underlies the inhibition of cell proliferation by GEM in pancreatic cancer. Given the previous findings that clinical efficacy does not require cytotoxic doses of GEM (7,8), we focused on the effects of lower concentrations of GEM.

Materials and methods

Cell line and cell cytotoxicity. We used the YPK-1 cell line, which was established from human pancreatic cancer (15,16). YPK-1 cells were maintained in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100 μ g/ml streptomycin. GEM was provided by Eli Lilly Research Laboratories (Indianapolis, IN, USA). MTT (assays of YPK-1 cells were performed to evaluate the cytotoxicity of GEM as described previously (14). The 50% inhibitory concentration (IC₅₀) at 72 h after treatment was 6.3×10^{-3} μ g/ml.

Cell growth assay of GEM. To investigate the effect of GEM on the proliferation of YPK-1 cells, we performed cell growth

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Key words: pancreatic cancer cell line, cDNA microarray, gemcitabine, cell cycle

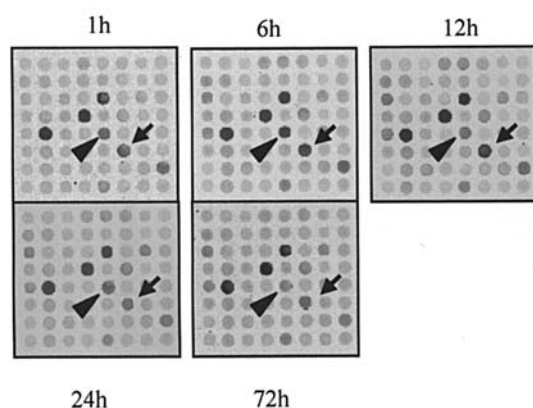


Figure 1. Representative images of colorimetric cDNA microarray analysis of YPK-1 cells exposed to half IC₅₀ GEM for 1, 6, 12, 24, and 72 h. Arrowheads and arrows indicate spots corresponding to HSPA8 and HNRPC, respectively. The images reveal temporal changes in gene expression.

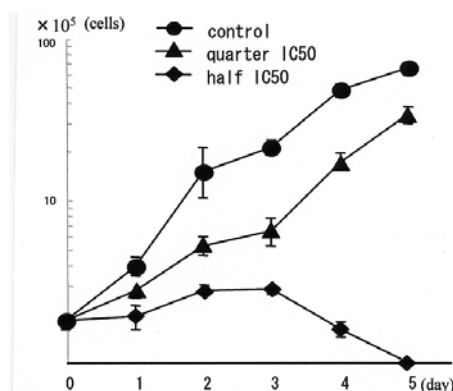


Figure 2. Effects of different concentrations of GEM on proliferation of YPK-1 cells. Cells were incubated with one of three concentrations of GEM: control (0 $\mu\text{g/ml}$), quarter IC₅₀ (1.6×10^{-3} $\mu\text{g/ml}$), and half IC₅₀ (3.2×10^{-3} $\mu\text{g/ml}$). The proliferation of cells was suppressed in a dose-dependent manner. The growth curves of cells incubated with the different concentrations of GEM were significantly different by one-way ANOVA ($p < 0.0001$).

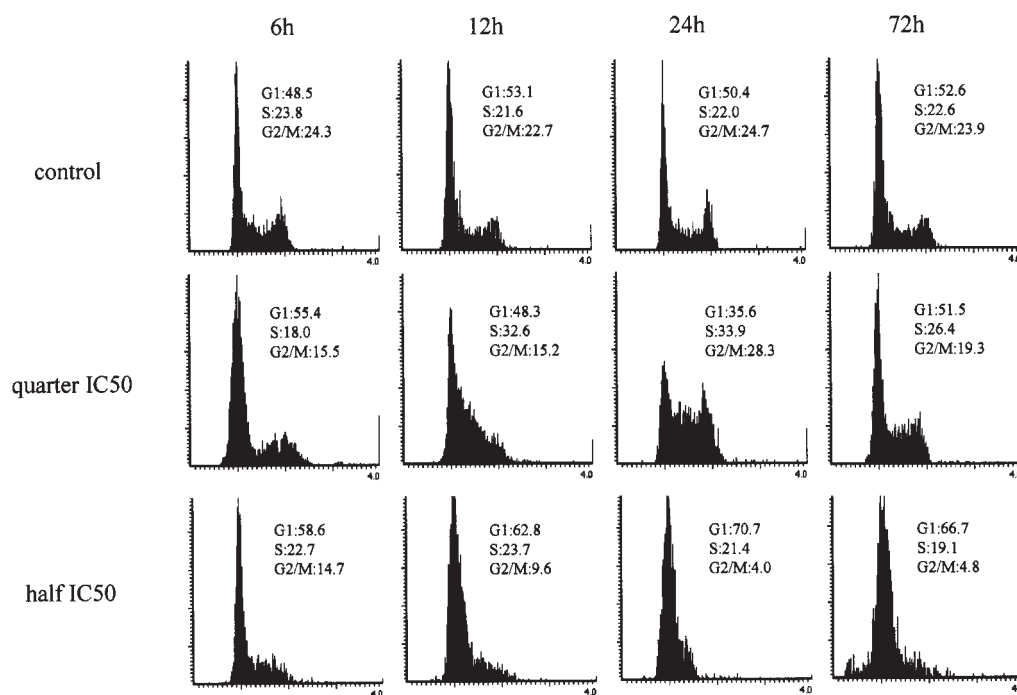


Figure 3. Temporal changes of the cell cycle in different concentrations of GEM. Cell cycle responses to the three concentrations of GEM were analyzed at 6, 12, 24, and 72 h after treatment. Cells exposed to quarter IC₅₀ GEM showed a block in late S phase, and the block appeared to be reversible at 72 h. Cells exposed to half IC₅₀ of GEM increased the number of cells in G1 phase at a relatively early time after treatment with GEM.

assays. YPK-1 cells were collected by trypsinization, and 1×10^5 cells were plated in 2×10 cm dishes. The cells were allowed to attach overnight and were then exposed to one of three concentrations of GEM, 0 $\mu\text{g/ml}$ (control), 3.2×10^{-3} $\mu\text{g/ml}$ (half IC₅₀), and 1.6×10^{-3} $\mu\text{g/ml}$ (quarter IC₅₀) for 5 days. The number of viable cells was determined by the trypan blue dye exclusion method. Experiments were performed in triplicate, and the mean values of the three experiments were calculated.

Cell cycle analysis. Cells cultured with the three different concentrations of GEM (control, half IC₅₀, and quarter IC₅₀)

were harvested at 6, 12, 24, and 72 h after treatment. Cells were stained with propidium iodide and analyzed by laser scanning cytometry (LSC) (LSC2; Olympus Co., Tokyo, Japan) as described previously (17).

cDNA microarray and data analysis. Differential gene expression in response to different concentrations of GEM was examined by cDNA microarray as described previously (14). Briefly, total cellular RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan) from GEM-exposed cells at 1, 6, 12, 24, and 72 h after treatment. Cy5-dUTP- and Cy3-dUTP- (Amersham Biosciences, Piscataway, NJ, USA) labeled probes

Table I. Primers of RPS8, SPARC, and GAPDH.

Gene symbol	Primer sequence	Number of PCR cycle	Product size (bp)
RPS8	5'-GCCGCATCCACACAGTCCGT-3' 3'-GCTTGGCTCCCTTCTTGCGG-5'	20	264
SPARC	5'-GGGACTAGAGGCTCAGTGGTG-3' 5'-GTCCCTAGAGCCCCTGAGAAG-3'	21	310
GAPDH	5'-GCATCCTGGGCTACACTGAG-3' 3'-ATCTGGGGAAGTTCTCCCCT-5'	18	336

were synthesized from total RNA per the manufacturer's protocol. We used a commercially available cDNA microarray, Human Chip version 1.0 (DNA Chip Research, Kanagawa, Japan), which contains 2976 cDNAs. The labeled probes were mixed with a hybridization solution. After hybridization, the slides were washed five times. Slides were scanned with a GenePix 4000 scanner (Axon Instruments, Union City, CA, USA). The fluorescence intensity of spot was compared to the local background, and background subtraction was performed. To normalize the data, the fluorescence ratio for each gene was adjusted to a median ratio value of all spots in the array of 1.0. Spots for which the ratio of the background-subtracted signal intensity to the local background was less than 3 were excluded from analysis. The ratios of each spot in duplicate experiments were averaged. Genes were categorized as temporal and significant profile changes if the ratio difference was 2.5-fold or greater for at least one time point, as shown in Fig. 1. Self-organizing map (SOM) analysis was performed with DNASIS Stat (DNA Chip Research). A three-row by three-column SOM was generated with an initial learning rate of 0.5 and an initial neighborhood size of 10.

RT-PCR analysis. To validate our microarray results for the SPARC and RPS8 genes, we carried out RT-PCR of these genes with the same RNAs used for the initial screening. The reverse transcriptase step was performed as described previously (14). Five microliters of cDNA solution (equivalent to the cDNA from 100 ng of initial RNA) was amplified in 45 μ l of PCR mixture containing 25 pmol of each primer for each target gene. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 61°C for 45 sec, and elongation at 72°C for 2 min. PCR protocols, primer sequences, and expected product sizes are shown in Table I. PCR products were separated by electrophoresis on 2.0% agarose gels and visualized by UV irradiation after ethidium bromide staining.

Statistical analysis. All data are presented as mean \pm SE. Statistical analyses were performed with repeated measures one-way analysis of variance (ANOVA) with StatView software (SAS Institute, Cary, NC, USA). Significance was defined as a p value of <0.05.

Results

Perturbation of cell proliferation by GEM. We first investigated how distinct concentrations of GEM affect the proliferation

of YPK-1 cells. The growth curves differed significantly between the two concentrations in a dose-dependent manner ($p < 0.0001$) (Fig. 2).

Influence of GEM on cell cycle progression. Changes in the cell cycle in response to different concentrations of GEM are shown in Fig. 3. A DNA histogram of control cells was almost unchanged from 6 h to 72 h after exposure of cells to GEM. Exposure of cells to quarter IC50 caused an accumulation of cells in the S phase between 12 and 24 h after exposure. This DNA histogram pattern had returned to normal by 72 h after exposure. In contrast, exposure of cells to half IC50 increased the number of cells in the G1 phase at 6 h after GEM exposure, and this was accompanied by a small sub-G1 peak at 72 h and appeared to lead into apoptosis.

Gene expression analysis by SOM in YPK-1 cells exposed to GEM. Genes were subjected to cluster analysis and a nine-partition SOM with a 3x3 algorithm (Fig. 4, Tables II and III). The resulting clusters were independent of the biological functions of the selected genes. The analysis revealed temporal changes in the expression of genes related to the cell cycle, transporter or immune response. The genes appeared to reflect the cellular response to GEM. We identified 24 genes whose expression altered in response to two different concentrations of GEM. The expression level of most genes was unchanged in cells exposed to either half IC50 or quarter IC50 of GEM for 24 h.

Validation of microarray data by RT-PCR. To validate the microarray data, we carried out RT-PCR of SPARC and RPS8, two genes selected randomly from the genes showing differential expression in response to GEM. The temporal changes in expression of the genes were consistent with the corresponding SOM patterns (Fig. 5). Thus, the microarray data were validated by the RT-PCR results.

Discussion

GEM blocks cell cycle progression in cells at the appropriate concentration (18). In the present study, G1 arrest was observed in cells exposed to half IC50 of GEM, and subG1 was observed in cells exposed to GEM for 72 h. It was anticipated that cells with suppressed proliferation would undergo apoptosis after 72 h (day 3). These observations were consistent with the findings of our growth assay (Fig. 1).

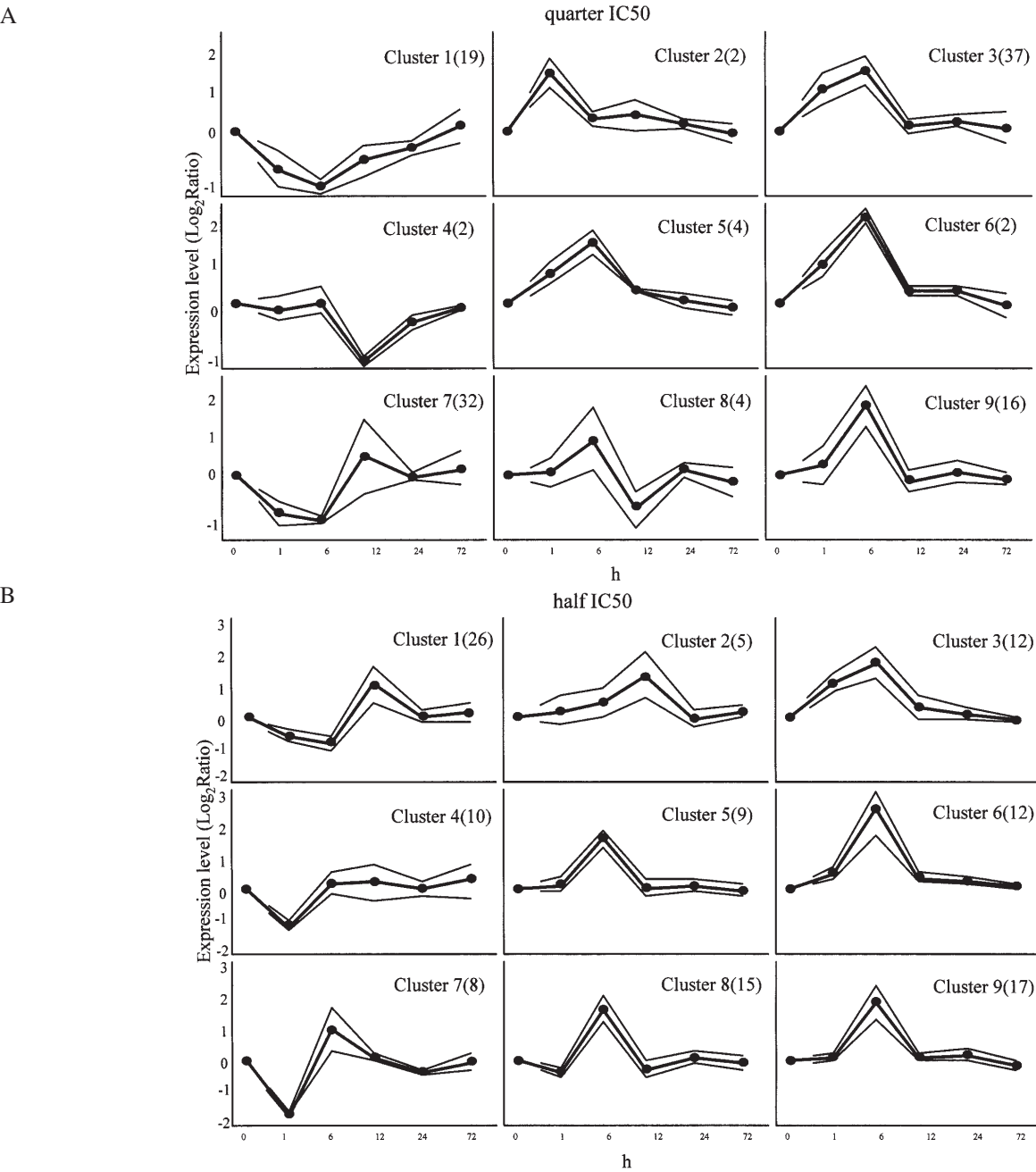


Figure 4. Nine-partition SOM showing temporal changes in expression in cells exposed to GEM. Approximately 90 genes for cells exposed to GEM (A, quarter IC₅₀; B, half IC₅₀) were identified with the criteria described above. Thick lines and dots indicate the average expression levels for each gene cluster. Thin lines indicate the standard error for each time point. The number in the top right corner of each panel represents the number of genes in the cluster.

Cells exposed to quarter IC₅₀ of GEM temporarily accumulated in late S phase, indicating that progression through S phase might be delayed. At 72 h of exposure, there was no significant difference between the quarter IC₅₀ and the control. Thus, a low concentration of GEM prolongs the S phase of YPK-1 cells. It is intriguing to investigate a set of genes linked with a difference in the cellular response to GEM. Thus, it is important to focus on the change of gene expression caused by different GEM concentrations.

Genes relevant to cell cycle regulation were identified by generation of SOMs for data from cells treated with GEM. In half IC₅₀-treated cells, CDKN1B, a cyclin-dependent kinase inhibitor implicated in the transition from proliferation to a quiescent state (19,20), was classified to cluster 2, and the

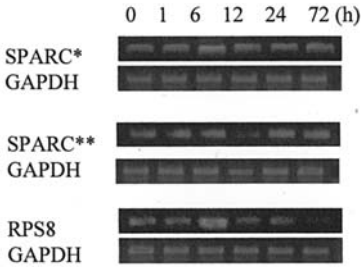


Figure 5. Validation of microarray data by RT-PCR. Temporal changes in expression of the SPARC and RPS8 genes were consistent with the corresponding SOM patterns. SPARC*, expression changes of cluster 8 in half IC₅₀; SPARC**, cluster 8 in quarter IC₅₀; and RPS8, cluster 6 in half IC₅₀. Glyceraldehyde-3-phosphate dehydro-genase (GAPDH) expression is shown as a control.

Table II. Temporal changed genes in half IC50.

Symbol	Unigene	Symbol	Unigene	Symbol	Unigene
Cluster 1					
NEDF	Hs.512608	MAN1B1	Hs.279881	PABPN1	Hs.117176
TAF15	Hs.402752	PPIA	Hs.356331	SERF2	Hs.424126
SOLH	Hs.416262	LYPLA2	Hs.413781	BCL2L1	Hs.305890
SMARCD2 ^a	Hs.250581	NYD-SP15	Hs.388220	CCT2	Hs.189772
GPSN2	Hs.306122	TAGLN2	Hs.406504	MSF	Hs.363475
SRRM2 ^a	Hs.433343	AP2A1	Hs.296426	IGKC	Hs.377975
GRN	Hs.180577				
Cluster 2					
PLA2G4C	Hs.18858	CDKN1B	Hs.238990	NUCKS ^a	Hs.510265
DDX21	Hs.169531	MSH6	Hs.445052		
Cluster 3					
TPT1 ^a	Hs.374596	HNRPC	Hs.476302	TRA1	Hs.192374
RPL7 ^a	Hs.421257	TAP1 ^a	Hs.352018	PA2G4	Hs.374491
PAI-RBP1	Hs.356427	RPL19 ^a	Hs.381061	IF2	Hs.158688
ALK ^a	Hs.410680				
Cluster 4					
HLA-B	Hs.77961	NOLC1 ^a	Hs.75337	GDI1	Hs.74576
HLA-A	Hs.181244	C2orf6	Hs.196437	COPZ1	Hs.181271
FXYD5	Hs.333418	GPX4	Hs.433951		
Cluster 5					
ACTB	Hs.426930	RPS27A	Hs.311640	HNRPD	Hs.438726
CAP1	Hs.104125				
Cluster 6					
PABPC1 ^a	Hs.387804	RPS8	Hs.512675	TCP1	Hs.4112
HNRPA1	Hs.356721	RPS3A ^a	Hs.356572	HSPA8 ^a	Hs.180414
HSPC016	Hs.356440	RPS6 ^a	Hs.408073	RPL5 ^a	Hs.469653
EIF3S3	Hs.127149				
Cluster 7					
MAT2A	Hs.77502	ANXA6 ^a	Hs.412117	PHGDH	Hs.3343
PSAP ^a	Hs.406455	PPAP2A	Hs.482121	HLA-F	Hs.411958
GNAI2	Hs.77269	FUS ^a	Hs.107720		
Cluster 8					
ATP1A1	Hs.371889	BZW1 ^a	Hs.355983	EEF1G	Hs.256184
SPARC ^a	Hs.111779	RALBP1	Hs.75447	ENO1	Hs.433455
ATF4	Hs.181243	SFRS5 ^a	Hs.166975	TEGT	Hs.35052
FTL	Hs.433670				
Cluster 9					
EEF1A1 ^a	Hs.439552	YWHAZ ^a	Hs.386834	EIF4A2	Hs.511904
ARPC5 ^a	Hs.126222	N4BP1	Hs.323712	UBE2V1	Hs.381025
H19	Hs.415722	DDX5	Hs.279806	EEF1A1L14 ^a	
GNAS	Hs.157307	ARHA	Hs.77273		

^aGene symbols are common in quarter IC50.

Table III. Temporal changed genes in quarter IC50.

Symbol	Unigene	Symbol	Unigene	Symbol	Unigene
Cluster 1					
GCN5L2	Hs.101067	C12ORF8	Hs.511762	HSCARG	Hs.288969
RPS2	Hs.498569	CAPZB	Hs.333417	YARS	Hs.239307
BST2	Hs.118110	ARHC	Hs.179735	MBTPS1	Hs.75890
PSAP ^a	Hs.406455	CYFIP2	Hs.211201	ANXA6 ^a	Hs.412117
RBBP2	Hs.76272	STAT1	Hs.21486	SFRS5 ^a	Hs.166975
PSMD1	Hs.3887	KIAA1161			
Cluster 2					
PSMB6	Hs.77060	EEF1B2			
Cluster 3					
TPT1 ^a	Hs.374596	RPL21	Hs.381123	RPS18	Hs.275865
TPI1	Hs.512711	VEGFC	Hs.79141	RPL12	Hs.408054
CHP	Hs.406234	PRDX1	Hs.180909	NTRK1	Hs.406293
RPS17	Hs.433427	CCT4	Hs.374334	RPLP1	Hs.356502
EEF1A1 ^a	Hs.439552	RPL7 ^a	Hs.421257	FTTH1	Hs.167344
UBB	Hs.356190	UREB1	Hs.326456	RPS5	Hs.378103
RPL26	Hs.406682	H2AFZ	Hs.119192	RPL19 ^a	Hs.381061
RPL32	Hs.265174	RPS24	Hs.356794	HMG2N	Hs.181163
KIAA1228	Hs.388073				
Cluster 4					
YWHAZ ^a	Hs.386834	BZW1 ^a	Hs.355983		
Cluster 5					
DDX39	Hs.311609	RTN4	Hs.436349	LAMR1	Hs.374553
Cluster 6					
PPP1R8	Hs.356590	ALK ^a	Hs.410680		
Cluster 7					
ZFP289	Hs.436204	GADD45B	Hs.110571	TLN1	Hs.375001
PTDSS2	Hs.12851	EIF4G2	Hs.183684	ACTN4	Hs.443619
SMARCD2 ^a	Hs.250581	FLJ13855	Hs.369120	KIAA0892	Hs.112751
PIGT	Hs.437388	SARS	Hs.444261	NDP52	Hs.154230
SRRM2 ^a	Hs.433343	FLJ20542	Hs.6449	SERHL	Hs.398085
NUCKS ^a	Hs.510265	ABCB1	Hs.21330	NOLC1 ^a	Hs.75337
PDCD11	Hs.239499	KIAA1533	Hs.365476	FUS ^a	Hs.107720
KIAA1171	Hs.353087				
Cluster 8					
ARPC5 ^a	Hs.126222	RPL9	Hs.412370	SH3BGR1	Hs.14368
SPARC ^a	Hs.111779				
Cluster 9					
PABPC1 ^a	Hs.387804	RPL13	Hs.410817	RPL13A	Hs.449070
HSPA8 ^a	Hs.180414	RPS3A ^a	Hs.356572	BTF3	Hs.446567
TAP1 ^a	Hs.352018	RPS6 ^a	Hs.408073	RPL5 ^a	Hs.469653
EEF1A1L14 ^a					

^aGene symbols are common in half IC50.

expression level slowly increased until 12 h after GEM exposure. Elevated expression of CDKN1B may cause G1 arrest of cells. Although other genes showed changes in expression levels in the present study, the roles of these genes in the anti-proliferative activity of GEM remain to be clarified.

Genes encoding transporters or proteins involved in immune responses showed altered expression more frequently in cells exposed to half IC50 than in cells exposed to quarter IC50. GPX4 encodes glutathione peroxidase 4, which protects cells against oxidative damage by anti-cancer drugs (21,22). ATP1A1 encodes an Na⁺/K⁺ transporter and is associated with the accumulation of drugs such as anti-cancer agents (23). The expression of ABCB1, an ABC transporter that influences sensitivity to anti-cancer agents (24), was temporarily decreased in cells exposed to quarter IC50. In contrast, the expression of genes encoding ribosome-related proteins was altered more frequently in cells exposed to quarter IC50 than in cells exposed to half IC50. The present data suggest that the expression of ribosomal proteins is linked to the susceptibility of tumor cells to chemotherapy (14,25,26). However, cellular responsiveness to a drug is dependent on the concentration of the drug because different genes are affected by different concentrations of drugs.

In the present study, 24 genes showed altered expression in response to both concentrations of GEM. In most of them, gene expression patterns in hierarchical cluster analysis were not affected by GEM concentrations (data not shown). It is likely that the changes in expression are not related to the inhibitory effects of GEM on cell proliferation. However, the level of expression of 2 of the 24 genes was dependent on the concentration of GEM. Temporal changes in the expression of these genes, SPARC (cluster 8 in half IC50 and cluster 8 in quarter IC50) and YWHAZ (cluster 9 in half IC50 and cluster 4 in quarter IC50), may be regulated by different molecular pathways that respond to varying concentrations of GEM. We believe that genes showing dose-dependent responses to GEM are associated with cell proliferation.

In conclusion, the gene expression profiles from cDNA microarray analysis coupled with SOM analysis revealed the molecular responses of YPK-1 cells to different concentrations of GEM. Identification of GEM-responsive genes provides insights into the anti-cancer mechanism of GEM and has the potential for clinical use to predict cellular responses to GEM. Further studies are needed to confirm that the genes identified in the present study are associated with growth inhibition in response to GEM.

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