Gene Expression Changes in a Chemoresistant Model with Human Esophageal Cancer Xenografts Using cDNA Microarray

TAKAHIRO YOSHIDA[1], TAKANORI MIYOSHI[1,2], JUN-ICHI SEIKE[1], HIROMICHI YAMAI[1], HIROKAZU TAKECHI[1], YASUHIRO YUASA[1] and AKIRA TANGOKU[1]

[1]*Department of Thoracic, Endocrine Surgery and Oncology, Institute of Health Bioscience, The University of Tokushima Graduate School, 2-50-1, Kuramoto-cho, Tokushima 770-8503;*
[2]*Department of Surgery, Tokushima Municipal Hospital, 2-34, Kitajousanjima-cho, Tokushima 770-0812, Japan*

Abstract. *Background: 5-Fluorouracil (5-FU) and cisplatin combined chemotherapy (FP) is commonly used for esophageal cancer. Acquired resistance needs to be overcome to improve the chemotherapeutic effect. Materials and Methods: The FP-resistant xenograft model using severe combined immunodeficient (SCID) mice was established as an acquired resistance model. RNA was extracted pretreatment, at the onset of the anticancer effect, during the most effective, and regrowth period in the FP administration group and during the mid-progressive period and the far advanced period in the control group. A microarray was applied to explore gene expression changes. Results: The data set containing up-regulated genes in the regrowth period was uploaded into Ingenuity Pathway Analysis. The expression change profiles suggested that activation of not only 5-FU- and cisplatin-specific genes, but also the Phosphoinositide 3-kinase (PI3K)/AKT signal were associated with FP resistance. Conclusion: A xenograft model using SCID mice with esophageal cancer cells would monitor gene changes during treatment and regrowth.*

Esophageal cancer is a malignant carcinoma with high invasiveness and metastasis, therefore, the prognosis is poor even after radical resection (1). The standard and active regimen for esophageal cancer is 5-fluorouracil (5-FU) and cisplatin combined chemotherapy (FP) (2). Clinical reports have shown that esophageal cancer, especially squamous cell carcinoma, responds well to chemotherapy and radiotherapy (3, 4). Recently, research concerning predictive factors for prognosis and chemoresponse has been performed and clinical application as tailor-made treatment is expected, however, the effect of anticancer drugs is not sufficient to improve the prognosis despite high response rates. Previous studies have not shown a significant difference in survival between responders and non-responders to anticancer drugs. One reason for this result is acquired resistance to anticancer drugs, therefore, overcoming acquired resistance is essential for improving the survival of patients treated with chemotherapy. Several researchers have already reported chemoresistant-related genes and the molecular mechanism of acquired resistance by clustering analysis of gene expression between sensitive and resistant cell lines in tissue and biopsy specimens from patients (5,6). It has been reported that genetic changes were associated with acquired resistance to the anticancer agent in cancer cell-line experiments (7), however, no reports have focused on expression change related to the chemoresistance of tumors during chemotherapy using cDNA microarray. Our previous studies have demonstrated that an orthotopically implanted thymic nude and severe combined immunodeficient (SCID) mouse model of human cancer cell lines was suitable for investigating metastases and anticancer drug effects (8-10). In this study, an acquired chemoresistant xenograft model was established using SCID mice with human esophageal cancer cell lines and a cDNA microarray analysis was conducted aimed at identifying changes in gene expression in the xenografts, in each period during treatment. The value of cDNA microarray in the FP-resistant xenograft model to elucidate complicated chemoresistant mechanisms was investigated.

Correspondence to: Takanori Miyoshi, MD, Ph.D., Department of Thoracic, Endocrine Surgery and Oncology, Institute of Health Bioscience, The University of Tokushima Graduate School, 2-50-1, Kuramoto-cho, Tokushima 770-8503, Japan. Tel/Fax: +81 886337143, +81 886337144, e-mail: tmiyoshi@clin.med.tokushima-u.ac.jp

Key Words: Chemoresistance, microarray, esophageal cancer.

Materials and Methods

Cell line. A human esophageal squamous cell carcinoma cell line (YES-3) was kindly provided by the Department of Surgery, Yamaguchi University, Japan. YES-3 was cultured in RPMI-1640 medium (Life Technologies, Inc., Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker,

Verviers, Belgium) and L-glutamine at 37˚C in a humidified atmosphere of 5% CO_2 and maintained in continuous exponential growth. For *in vivo* inoculation, the cells were harvested from the culture flasks after brief trypsinization. Only single-cell suspensions of >95% viability (trypan blue exclusion) were injected.

Chemicals. The 5-FU was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and was dissolved in distilled water just before use. Cisplatinum (CDDP) was provided by Nihon Kayaku Co., Ltd. (Tokyo, Japan) and was dissolved in sterile 0.85% NaCl.

SCID mouse subcutaneous xenograft model and tumor size measurement. SCID mice, 8-10 weeks old (Fox CHASE C.B. 17/Icr-Scid Jcl) were obtained from CLEA Japan, Inc (Tokyo, Japan) and maintained under specific pathogen-free conditions. Tumor cell suspensions were concentrated to 1×10^6 cells/ml phosphate-buffered saline (PBS) and injected into the subcutaneous dorsal region of the mice using a 26-gauge needle (whole injection volume, 0.3 ml/mouse). The SCID mice with xenografts were divided into a 5-FU/CDDP administration group and a control group. The anticancer drugs and solvents were administered when the xenografts became measurable. The tumor volume and body weight were measured once a week from the start of drug administration. The tumor weight was calculated according to the following formula: estimated tumor weight (g)=width $(mm)^2\times$length (mm)/2. The body weight average of each group was measured once a week before the drug administration for determining dosage of 5-FU and CDDP. The anticancer drugs and control solvent were administered repeatedly throughout the experimental period. Xenograft tissue with a volume adequate for cDNA microarray was harvested pretreatment (FP0), at the onset of treatment effect (FP1), when the treatment was most effective (FP2) and after regrowth (FP3) and in the control group pretreatment (FP0) and at the mid-progressive (C1) and far advanced (C2) periods just before the first administration at pretreatment and 24 h later after the final administration during treatment, on ice and immediately stored in RNA isolation (ISOGEN, Nippon Gene Co., Tokyo, Japan) at –80˚C until cDNA microarray analysis.

Treatment schedule. The 5-FU/CDDP group (FP group) was administered 5-FU, 20mg/kg body weight, 5 days a week orally and CDDP, 2.5 mg/kg body weight, twice a week i.p. The control group was administered distilled water, 0.2 ml/mouse orally and 0.85% NaCl, 0.4 ml/mouse i.p. (11).

Construction and analysis of cDNA microarray. The total RNA was extracted from the frozen samples of each xenograft using ISOGEN according to the manufacturer's instructions. The samples from each xenograft were analyzed by oligonucleotide microarray. The Applied Biosystems Human Genome Survey Array contains 32332 60-mer oligonucleotide probes representing a set of 29098 individual human genes and more than 1,000 24-mer control probes. The 60-mer oligo probes were synthesized using standard phosphoramidite chemistry and solid-phase synthesis, and quality controlled by mass spectrometry. A 24-mer oligo internal control probe (ICP) was co-spotted with every 60-mer gene expression probe on the microarray. Each sample was reverse transcribed in the presence of Cy5-labeled deoxycytidine 5'-triphosphate (dCTP). Digoxigenin-uridinetriphosphate (UTP)-labeled cRNA was generated and linearly amplified from 1 μg of total RNA using an Applied Biosystems Chemiluminescent RT-IVT Labeling Kit v 2.0 (Applied Biosystems, Foster City, CA, USA) and the manufacturer's protocol. Array hybridization (one array per sample), chemiluminescence detection, image acquisition and analysis were performed using an Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol (12). First, 10 μg of labeled cRNA targets were fragmented into 100-400 bases by incubating with fragmentation buffer, mixed with internal control target: ICT, 24-mer oligo labeled with fluorescent dye (LIZ® dye) and hybridized to each microarray. Four images, a spot, short or long single color-chemiluminescent image, were used for gene expression analysis. The images were auto-grided and chemiluminescent signals of a single color were quantified, corrected for background and spot and spatially normalized.

Gene expression analysis. The cut-off value was set at 0.5 to 2.0 for the ratio (more than 2.0: up-regulation, 0.5-2.0: no change, less than 0.5: down-regulation) and genes with a signal/noise ratio <3 in all the xenografts were excluded from additional investigation.

Gene network analysis. The data were analyzed using Ingenuity Pathway Analysis (IPA) (13). The data containing named FP-resistant-related genes and their corresponding expression fold changes were uploaded into the application. The genes in this database, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated, based on their connectivity. The functional analysis of IPA identifies the biological functions and/or diseases that are most significant to the data set. The genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The nodes are displayed using various shapes that represent the functional class of the gene product. Various labels are used to describe the nature of the relationship between nodes. (*e.g.*, P, phosphorylation, T, transcription).

Canonical pathway analysis. The canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set which contained the named up-regulated genes and normalized signal ratios between the most effective period and the regrowth period. The significance of the association between the data set and the canonical pathway was measured in two ways. The ratio of the number of genes from the data set that mapped to the canonical pathway was divided by the total number of genes that mapped to the canonical pathway and the Fisher's exact test was used to calculate the *p*-value determining the probability that the association between the genes in the data set and the canonical pathway was explained by chance alone (13).

Results

Xenografts in the course of treatment. The weight changes of the xenografts of the FP and control groups are shown in Figure 1. The weight loss of the xenografts in the FP group was largest on day 15 and smallest on day 29. The

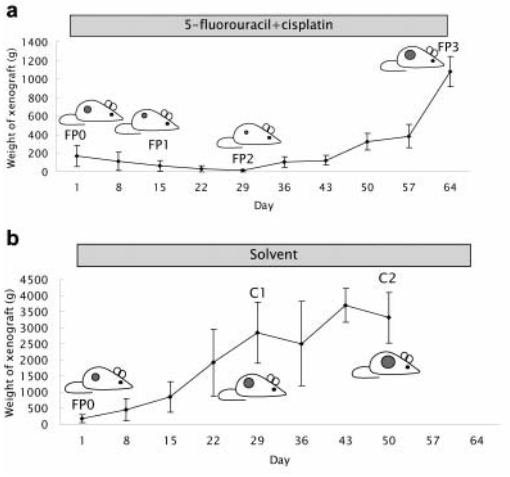

Figure 1. *Weights of xenografts of SCID mice during treatment. (a) Treated with 5-fluorouracil and cisplatin. FP0: pretreatment, FP1: onset of effect, FP2: treatment most effective and FP3: regrowth. (b) Treated with solvent, the control group. FP0: pretreatment, C1: mid-progressive, C2: far advanced.*

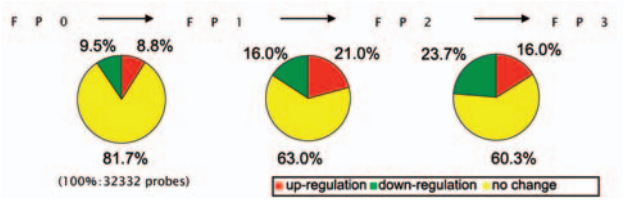

Figure 2. *Proportion of genes regulated during treatment and tumor growth. FP0: pretreatment, FP1: onset of effect, FP2: treatment most effective, FP3: regrowth.*

xenografts showed regrowth from day 36 which was rapid from day 57 to day 64 despite the administration of anticancer drugs (Figure 1a). The xenografts in the control group represented natural progress, and tended to increase, even though the growth of the weight of xenografts was limited by tumor necrosis from day 29 (Figure 1b).

Gene expression change profiles. The gene expression change profiles in the FP group were investigated by comprehensive microarray analysis. Out of the 32332 probes, the number of up-regulated genes in each period of FP0→FP1, FP1→FP2 and FP2→FP3 was 2831 (8.8%), 6780 (21.0%) and 5177 (16.0%), respectively. The number of down-regulated genes in the same period was 3085 (9.5%), 5157 (16.0%) and 7654 (23.7%), respectively. Out of these 5177 genes up-regulated in the period of FP2→FP3, 3165 genes (61.1%) were down-regulated, only 112 genes (2.2%) were up-regulated and 1900 genes (36.7%) showed no change in the FP1→FP2 period. It was interesting that the majority of these 5177 genes up-regulated in the regrowth period had been down-regulated during the FP1→FP2 period. In the control group, the number of up-regulated genes in the periods FP0→C1 and C1→C2 was 2688 (8.3%), and 2165 (6.7%), and the number of down-regulated genes in the same periods was 1863 (5.8%) and 3043 (9.4%), respectively. Thus, more genes showed significant change in the FP group than in the control group (Figure 2).

Ingenuity Pathway Analysis. The 2620 genes which were up-regulated significantly with > 3 signal/noise ratio comparing FP2 with FP3 (FP2→FP3) were selected as FP-resistant related genes. These 2620 genes and their ratio of normalized signal in FP3/FP2 were subjected to IPA. This analysis suggested that out of 164 canonical pathways, pyrimidine metabolism, G2/M DNA damage checkpoint regulation, purine metabolism and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway played an important role in the resistance to anticancer drugs (Figure 3). In the molecular network of the PI3K/AKT signaling pathway several areas up- and down-stream were activated (Figure 4).

Expression changes profiles of novel genes during chemotherapy. Genes were selected from the data, focusing on novel genes associated with drug resistance with different functions, such as ATP-binding cassette (ABC) transporter: ATP-binding cassette, sub-family B, member 1 (MDR1), ATP-binding cassette, sub-family C, member 1 (MRP1), 5-FU-specific resistant-related genes: thymidylate synthase (TYMS), dihydropyrimidine dehydrogenase (DPYD), uridine monophosphate synthase (UMPS), CDDP-specific resistant-related genes: non-metastatic cells 1 (NME1), excision repair cross-complementing 1 (ERCC1), and the PI3K/AKT signaling pathway (upper stream: erythroblastic leukemia viral oncogene homolog 2 (ERBB2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), main stream: PIK3 catalytic beta polypeptide (CB), AKT downstream: nuclear factor kappa B1 (NFKB1), tumor protein p53 (TP53). The gene expression changes recorded as the ratio of normalized signals between xenografts, were divided into up-regulation, no change, and down-regulation. The gene expression changes during administration were analyzed and are shown in Figure 5. The ratio of these changes was also expressed as (normalized signal in FP3) / (normalized signal in FP2) of the AKT1 gene, which was 3.059 during the regrowth period (from FP2 to FP3). The genes which were down-regulated from FP1 to FP2 and up-regulated from FP2 to FP3 were considered to be related to FP-resistance and are underlined.

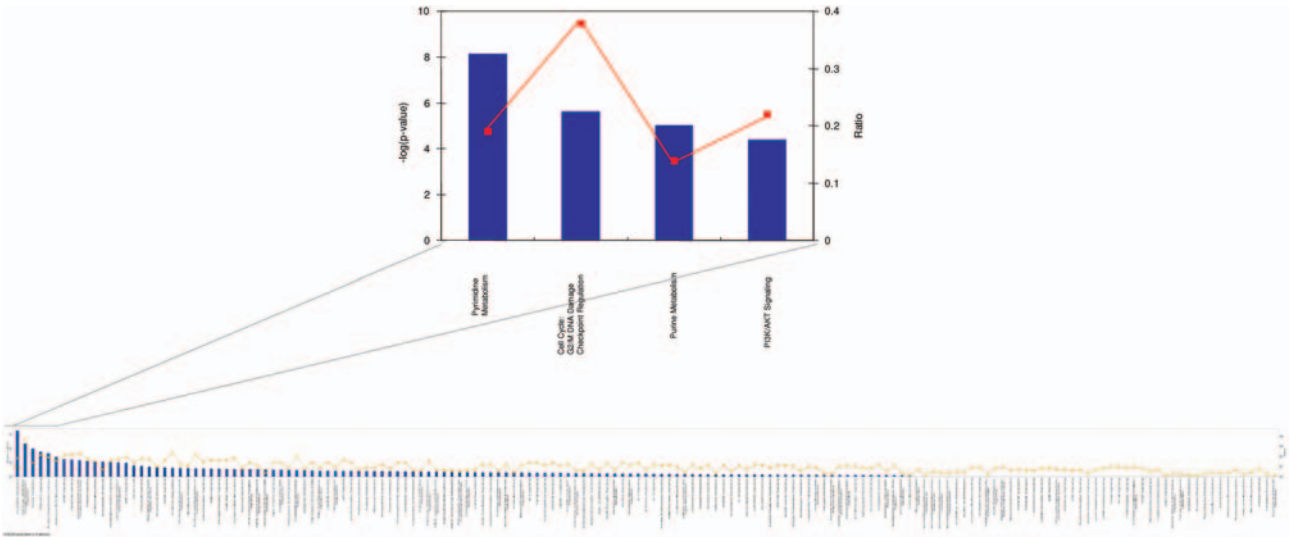

Figure 3. *Canonical Pathway Analysis of the up-regulated genes in the FP2→FP3 period showing the four most important pathways related to acquired chemoresistance in terms of significance (–log[p-value]) and ratio. Significance (bar graph): –log of p-value calculated by Fisher's exact test for genes in the data set and the canonical pathway. Ratio (dot): the number of genes from the data set in the canonical pathway/total number of genes that mapped to the canonical pathway. *data set: gene names and normalized signal ratios up-regulated in the FP2→FP3 period.*

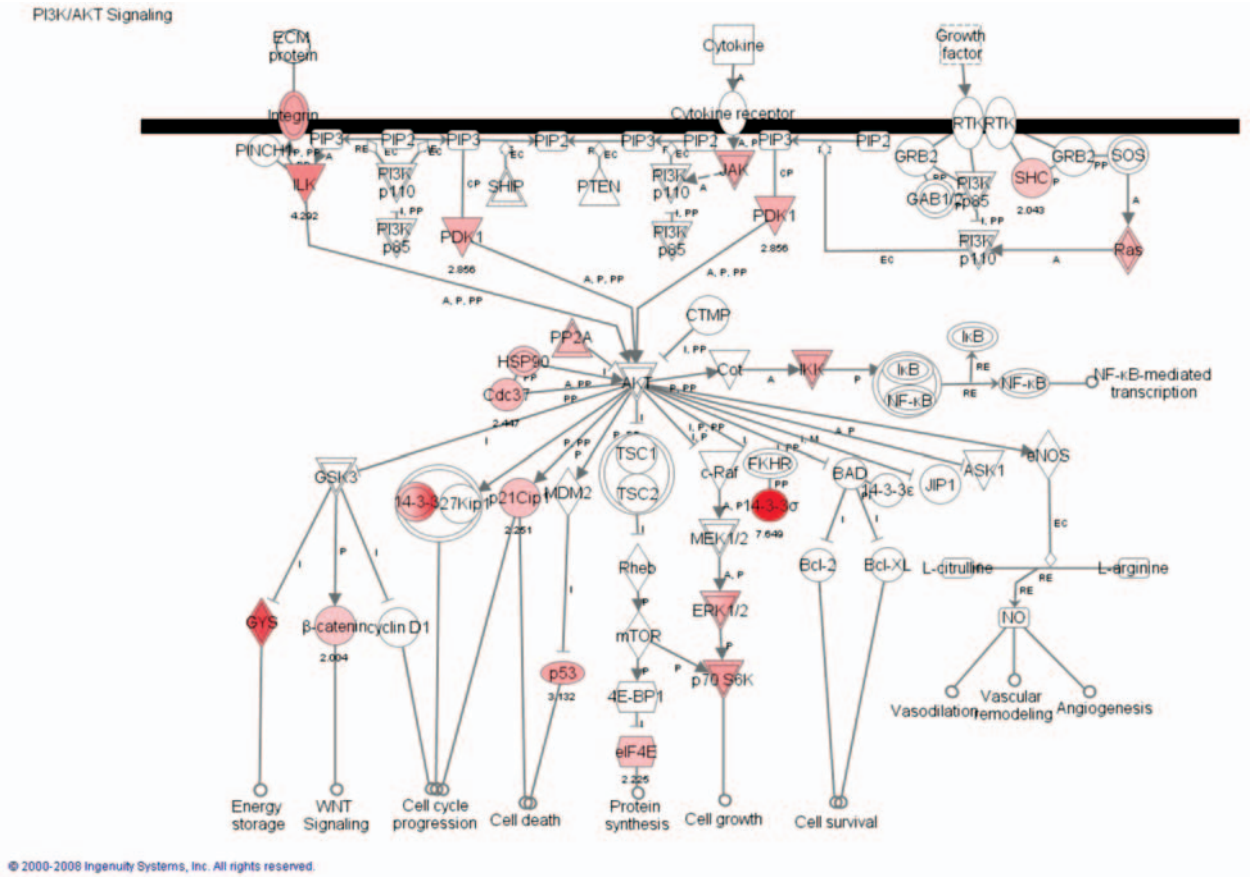

Figure 4. *Molecular network of PI3K/AKT signaling pathway and up-regulated genes in the FP2→FP3 period. Red nodes represent up-regulated molecules based on a data set of up-regulated genes in the molecular network of the PI3K/AKT signaling pathway. The intensity of the node color indicates the degree of up-regulation.*

Discussion

Acquired resistance of a tumor leads to regrowth during anticancer agent treatment, even if the tumor initially responds to the anticancer drugs. Many previous studies have reported a change of gene expression and the level of protein expression in human cancer tissues and cell lines between pre- and post-chemotherapy (5, 6), and microarray analyses were performed to identify the genes related to sensitivity and resistance to anticancer drugs (14, 15). The present study reports a new method for extracting chemoresistant-related genes using microarray analysis by measuring the gene expression changes of xenografts during chemotherapy up to regrowth. This *in vivo* human esophageal cancer xenograft study, using an SCID mouse model, might elucidate a resistance mechanism without immune influence. The results of the present analysis supported previous reports of chemoresistance-related genes and the PI3K/AKT signaling pathway as an important mechanism associated with acquired resistance to anticancer drugs. 5-FU and CDDP are key drugs in esophageal cancer and evidence is increasing to support a drug-resistant mechanism by the amplification of genes encoding TYMS, a target enzyme of 5-FU and DPYD, which is important in the resistance mechanism (16, 17).

Expression changes of TYMS did not occur in this study during treatment or tumor progression. DPYD and ERCC1, drug-specific sensitivity-related genes for 5-FU and CDDP, were down-regulated in the effective period (FP1→FP2) and up-regulated in the regrowth period (FP2→FP3). The data showed that DPYD and ERCC1 are associated with acquired resistance to 5-FU and CDDP in esophageal cancer patients. In addition, amplification of the genes (MDR1, MRP1) to encoding one of the ATP-binding cassette transporters associated with multi-drug resistance has been well documented (18, 19) and gene expression changes were recognized in MRP1, ATP-binding cassette, sub-family C, member 3 (ABCC3) and ABCC5 in the present study. Among 24 novel genes, a few were regulated during the natural progression in the control group, while the majorities were regulated during drug administration in the FP group. Moreover, the majorities of these genes were down-regulated in the effective period (FP1→FP2) and up-regulated in the regrowth period (FP2→FP3). An important molecule mammalian target of rapamycin (mTOR) activates the PI3K/AKT signaling pathway and induces cell proliferation and anti-apoptosis, and rapamycin as an inhibitor of mTOR has already been developed (20). Up-regulation of the mTOR gene in the regrowth period was not shown in the present study, but should be examined for its relationship with the resistance mechanism. Not only the change of anticancer drug-specific sensitivity factors, but also the activation of survival signals, such as the PI3K/AKT signaling pathway, were related to the resistance to the anticancer drugs. Furthermore, a survival signal might play an important role in malignant alteration during the administration of anticancer drugs.

Group		Probe ID	UniGene ID	Gene Symbol	FP0→FP1	FP1→FP2	FP2→FP3	FP0→C1	C1→C2
PI3K/AKT signaling pathway related gene	up-stream	136952	Hs.488293	EGFR	0,797	1,712	0,635	1,000	1,123
		105627	Hs.446352	ERBB2	1,091	0,431	2,186	0,870	0,706
		157943	Hs.73793	VEGF	1,028	2,152	0,887	0,843	2,478
		227323	Hs.78781	VEGFB	1,275	0,372	2,473	0,767	1,285
		170337	Hs.435215	VEGFC	1,556	0,361	3,536	0,400	3,119
	PI3K/AKT	149852	Hs.525622	AKT1	1,633	0,390	3,059	1,035	1,241
		121831	Hs.515406	AKT2	0,389	2,736	0,920	0,727	0,727
		190556	Hs.498292	AKT3	0,715	1,984	0,228	0,889	0,725
		205200	Hs.239818	PIK3CB	1,710	0,169	3,368	0,928	1,039
		138389	Hs.371344	PIK3R2	1,119	0,709	1,091	0,644	0,642
	down-stream	107316	Hs.500466	PTEN	1,332	0,633	1,078	0,799	1,519
		100942	Hs.338207	mTOR	1,873	0,564	1,743	1,244	1,310
		158269	Hs.408312	TP53	0,591	0,376	3,132	0,892	0,617
		180365	Hs.515536	RRAS	0,698	0,387	3,140	0,502	1,380
		202363	Hs.431926	NFKB1	1,233	0,810	1,305	0,829	1,434
5-fluorouracil-specific gene		154415	Hs.369762	TYMS	1,340	0,612	1,602	0,867	1,298
		112355	Hs.335034	DPYD	0,975	0,490	2,520	1,669	0,546
		132709	Hs.2057	UMPS	1,278	0,331	3,677	0,936	1,049
Cisplatin-specific gene		182774	Hs.118638	NME1	1,350	0,802	2,150	0,814	1,514
		116205	Hs.435981	ERCC1	1,376	0,451	2,954	0,990	1,062
ABC transporter-related gene		117528	Hs.391464	ABCC1(MRP1)	1,741	0,291	3,325	0,902	1,192
		109767	Hs.463421	ABCC3	1,740	0,316	4,061	0,649	1,590
		197753	Hs.368563	ABCC5	1,291	0,253	2,981	1,014	0,826
		182279	Hs.489033	ABCB1(MDR1)	0,518	0,997	1,040	0,520	1,536

Figure 5. *Expression change profiles of novel genes during chemotherapy. Number in a box represents the ratio of normalized signal between the FP and control group xenografts. Red: up-regulation, green: down-regulation, yellow: no change.*

In conclusion, in a xenograft model during chemotherapy many genes are down-regulated during the effective period of anticancer agents and many genetic up-regulations are seen during the regrowth period including not only anti-cancer drug-specific genes, but also survival signals. Hence, the prediction of chemoresistance-related genes and understanding of the organization of the resistance mechanism may lead to the inhibition of acquired resistance and enhance the power of anticancer agents.

References

1 Ando N, Ozawa S, Kitagawa Y, Shinozawa Y and Kitajima M: Improvement in the results of surgical treatment of advanced squamous esophageal carcinoma during 15 consecutive years. Ann Surg *232*: 225-232, 2000.

2 Enzinger PC, Ilson DH and Kelsen DP: Chemotherapy in esophageal cancer. Semin Oncol *26*: 12-20, 1999.

3 Stahl M, Stuschke M, Lehmann N, Meyer HJ, Walz MK, Seeber S, Klump B, Budach W, Teichmann R, Schmitt M, Schmitt G, Franke C and Wilke H: Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. J Clin Oncol *23*: 2310-2317, 2005.

4 Law S, Fok M, Chow S, Chu KM and Wong J: Preoperative chemotherapy versus surgical therapy alone for squamous cell carcinoma of the esophagus: a prospective randomized trial. J Thorac Cardiovasc Surg *114*: 210-217, 1997.

5 Chan HS, Haddad G, Thorner PS, DeBoer G, Lin YP, Ondrusek N, Yeger H and Ling V: P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med *325*: 1608-1614, 1991.

6 Schneider S, Uchida K, Brabender J, Baldus SE, Yochim J, Danenberg KD, Salonga D, Chen P, Tsao-Wei D, Groshen S, Hoelscher AH, Schneider PM and Danenberg PV: Down-regulation of TS, DPD, ERCC1, GST-Pi, EGFR, and HER2 gene expression after neoadjuvant three-modality treatment in patients with esophageal cancer. J Am Coll Surg *200*: 336-344, 2005.

7 Saito T, Hikita M, Kohno K, Tanimura H, Miyahara M and Kobayashi M: Enhanced expression of the multidrug resistance gene in vindesine-resistant human esophageal cancer cells. Oncology *51*: 440-445, 1994.

8 Miyoshi T, Kondo K, Ishikura H, Kinoshita H, Matsumori Y and Monden Y: SCID mouse lymphogenous metastatic model of human lung cancer constructed using orthotopic inoculation of cancer cells. Anticancer Res *20*: 161-163, 2000.

9 Kondo K, Fujino H, Miyoshi T, Ishikura H, Sakiyama S and Monden Y: Orthotopically implanted SCID mouse model of human lung cancer suitable for investigating metastatic potential and anticancer drug effects. Oncol Rep *12*: 991-999, 2004.

10 Fujino H, Kondo K, Miyoshi T, Ishikura H, Takahashi Y, Sawada N, Hirose Y, Takizawa H, Nagao T, Sakiyama S and Monden Y: Establishment of patient-like SCID mouse model by orthotopically implanting primary cultured cells from surgically-resected lung cancer tissues. Oncol Rep *10*: 1709-1715, 2003.

11 Wada Y, Yoshida K, Suzuki T, Mizuiri H, Konishi K, Ukon K, Tanabe K, Sakata Y and Fukushima M: Synergistic effects of docetaxel and S-1 by modulating the expression of metabolic enzymes of 5-fluorouracil in human gastric cancer cell lines. Int J Cancer *119*: 783-91, 2006.

12 Wang Y, Barbacioru C, Hyland F, Xiao W, Hunkapiller KL, Blake J, Chan F, Gonzalez C, Zhang L and Samaha RR: Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. BMC Genomics *7*: 59, 2006.

13 Ingenuity®RSystems, www.ingenuity.com

14 Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, Scudiero DA, Eisen MB, Sausville EA, Pommier Y, Botstein D, Brown PO and Weinstein JN: A gene expression database for the molecular pharmacology of cancer. Nat Genet *24*: 236-244, 2000.

15 Suganuma K, Kubota T, Saikawa Y, Abe S, Otani Y, Furukawa T, Kumai K, Hasegawa H, Watanabe M, Kitajima M, Nakayama H and Okabe H: Possible chemoresistance-related genes for gastric cancer detected by cDNA microarray. Cancer Sci *94*: 355-9, 2003.

16 Fujiwara H, Terashima M, Irinoda T, Takagane A, Abe K, Nakaya T, Yonezawa H, Oyama K, Takahashi M, Saito K, Takechi T, Fukushima M and Shirasaka T: Superior antitumour activity of S-1 in tumours with a high dihydropyrimidine dehydrogenase activity. Eur J Cancer *39*: 2387-2394, 2003.

17 Takechi T, Fujioka A, Matsushima E and Fukushima M: Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells. Eur J Cancer *38*: 1271-1277, 2002.

18 Takara K, Obata Y, Yoshikawa E, Kitada N, Sakaeda T, Ohnishi N and Yokoyama T: Molecular changes to HeLa cells on continuous exposure to cisplatin or paclitaxel. Cancer Chemother Pharmacol *58*: 785-793, 2006.

19 Norris MD, Bordow SB, Marshall GM, Haber PS, Cohn SL and Haber M: Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. N Engl J Med *334*: 231-238, 1996.

20 Rubio-Viqueira B and Hidalgo M: Targeting mTOR for cancer treatment. Curr Opin Investig Drugs *7*: 501-512, 2006.

Received November 19, 2008
Revised January 19, 2009
Accepted February 26, 2009