Dynamic alteration of gene expression induced by anticancer-agent exposure in gastric cancer cell lines

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Abstract. The prognosis for advanced gastric cancer remains very poor due to the limited effectiveness of chemotherapy agents. The range in efficacy of these chemotherapy agents is likely the result of various factors including gene expression. In addition, the emergence of drug resistance during chemotherapy can result in the undesirable outcome of patients experiencing the side effects of chemotherapy without benefiting in terms of tumor response. Gene expression profiling provides a unique opportunity to gain insight into the development of different types of gastric cancer. We applied the TaqMan low density array to investigate the expression of 96 genes in four gastric cancer cell lines (TMK-1, MKN-45, MKN-74 and CDDP-resistant MKN-74). The cell lines displayed different patterns of gene expression alteration after exposure to the chemotherapeutic drugs cisplatin and 5-fluorouracil. We also established that cisplatin-resistant MKN-74 cells do not show the same gene expression patterns as the parental MKN-74 cell line at 0, 8 and 24 h after CDDP exposure. This study reveals the underlying complexity of biological and pathological events and could, perhaps, contribute to new experimental protocols or treatments that would achieve a consistent and desirable outcome of chemotherapy.

Introduction

Gastric cancer is the fourth most common cancer world-wide (1). Although early gastric cancer, which is defined as a tumor confined to the mucosa or submucosa, has a 5-year survival rate exceeding 90% in Japan, the prognosis for advanced gastric cancer (AGC) remains poor. Surgical resection is the most powerful tool to overcome AGC, and recent studies suggest that systemic chemotherapy after surgery might improve the prognosis of AGC patients (2). A previous study showed that chemotherapy for metastatic gastric cancer yields a significant survival benefit, compared with that of best support care without chemotherapy (3).

Cisplatin (CDDP) and 5-fluorouracil (5-FU) are the key therapeutic agents for AGC; whereas, chemotherapy with single agents only achieves a response rate of 10-20% (4,5) in AGC. The difference between an effective and an ineffective response to anticancer drugs in AGC is likely dependent on various factors including polymorphisms, gene expression profiles in cancer cells (6,7) and several host factors. In addition, the emergence of drug resistance after chemotherapy can result in an undesirable outcome in AGC because many cancer patients suffer from the adverse effects of chemotherapy without any benefit in terms of tumor response.

Theoretically, the two major mechanisms of drug resistance are i) intrinsic resistance, in which untreated tumor cells are inherently insensitive to the chemotherapeutic agent and ii) acquired resistance, in which treated tumor cells become insensitive after drug exposure (8). It is well known that acquired drug resistance is caused by many factors such as host metabolism, the genetic nature of cancer cells or epigenetic changes of cancer cells, accompanied by numerous molecular events (9).

Several studies have recently suggested that there are dynamic alterations in gene expression evoked by several exogenous stimulators such as cytokines or chemotherapeutic agents in normal and cancer cells (10-14) and drug sensitivity/resistance has been predicted by measuring baseline expression levels of chemoresistance-related genes without such exogenous stimulation. The dynamics of gene expression should be interpreted in context with all biological behaviors in normal and cancer cells. An understanding of the changes in gene expression in cancer cell lines with and without treatment could shed light on the complexity of biological and pathological processes, perhaps leading to successful experimental protocols or treatments that produce a desirable outcome with, for example, chemotherapy.

The Taqman low density array system used in this study is a novel tool for quantifying gene expression (15,16). This system enables simple and precise measurement of gene

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expression that is equivalent to standard quantified PCR levels; additionally, multiple genes can be analyzed without the difficulty of quantifying differences between two experimental time points, a downside of traditional microarrays. Herein, we demonstrate the dynamics in gene expression among the three kinds of human gastric cancer cell lines, which were treated with either CDDP or 5-FU. In addition, a newly established CDDP-resistant MKN-74 cell line treated with CDDP is also evaluated and compared to the dynamics of the parental cell line MKN-74 with CDDP treatment. This study demonstrates that various gene expression dynamics occur in gastric cancer, and each pattern reflects a unique biological event that could have implications for treatment outcome.

Materials and methods

Cell lines and anticancer agents. Three human gastric cancer cell lines (TMK-1, MKN-45 and MKN-74) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in RPMI-1640 (Gibco BRL, Gaithersburg, MD, USA) with 1% antibiotics and 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). All cells were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. All experiments were performed using exponentially growing cells. The anticancer agent 5-FU was purchased from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). CDDP was purchased from Nihon Kayaku Co. Ltd. (Tokyo, Japan). In addition, CDDP-resistant MKN-74 cells were also established after a long period of incubation (3-6 months) with low dose (1-10 μg/ml) CDDP.

MTT assay. We evaluated the *in vitro* cytotoxic effects of 5-FU and CDDP on the cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay as reported by Mosmann with some modifications (17-19). Control wells contained 100 μl cell suspension and 100 μl RPMI-1640 containing 10% FBS. Plates were incubated for 48 h at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2. A mixture of 0.4% MTT and 0.1 M sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka, Japan), each dissolved in 10 μl phosphate-buffered saline and filtered through 0.45 μm membrane filters (Millipore, Bedford, MA, USA), was then added, and the plates were incubated for an additional 3 h at 37˚C. After the final incubation, 150 μl dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) was added to each well to dissolve the MTT-formazan salt, and the plates were shaken mechanically for 10 min on a mixer (Model 250, Sonifier, Branson, MO, USA). Optical densities of each well were determined on a model EAR 340 easy reader (SLT-Lab Instruments, Salzburg, Austria) at 540 and 630 nm. Survival ratio and inhibition ratio were calculated as absorbance of treated cells/absorbance of control cells and 1-survival ratio, respectively.

Determination of 50% inhibiting concentration (IC_{50}) of CDDP/5-FU for each cell line. We evaluated IC_{50} values of 5-FU/CDDP for all 4 cell lines in culture by MTT assay. MKN-45 cells ($3x10^3$) or TMK-1 cells ($5x10^3$) or MKN-74 cells were cultured using 96-well microplates in RPMI-1640 medium containing 10% FBS for 24 h and exposed to various concentrations of CDDP or 5FU for 48 h to calculate the IC_{50} of CDDP/5FU for each cell line with a concentration-survival curve.

Cell sampling, RNA extraction and gene expression analysis using the TaqMan low density array. Each cancer cell line was exposed with IC_{50} of 5-FU and CDDP, as previously described, and sampled at 0, 8 and 24 h after drug incubation. The TaqMan low density array (Applied Biosystems, Foster City, CA, USA) was applied to investigate the expression of 96 genes in the 4 gastric cancer cell lines (Table I). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using the Applied Biosystems micro fluidic card according to the manufacturer's instructions. In brief, 2.5 μg of total RNA was reverse transcribed using the High Capacity cDNA archives kit and MultiScribe reverse transcriptase (Applied Biosystems). The reaction mixtures were incubated initially at 25˚C for 10 min and subsequently at 37˚C for 120 min. Quantitative PCR (TaqMan) assays were performed using the Micro fluidic card system incorporating Assays-On-Demand (Applied Biosystems), a pre-validated library, into 384-well micro fluidic cards. One hundred ng of cDNA samples, along with 50 μl of 2x PCR master mix, were loaded into each channel on the micro fluidic card, followed by a brief centrifugation. The card was then sealed, and real-time PCR and relative quantification were performed using an ABI PRISM 7900 sequence detection system (Applied Biosystems). The expression of each gene was normalized using ß-actin as a reference, and the relative expression levels were qualified using the ΔCt method (Applied Biosystems) (20). All RNA extractions were carried out in triplicate. Data are expressed as average ratio (n=3) ralative control.

Statistical analysis. Statistical analysis was performed using Student's t-test, and a significance level of $P<0.05$ was regarded as statistically significant.

Results

IC_{50} values of CDDP/5-FU for each cell line. IC_{50} values of CDDP were calculated as 7.9, 18.7, 23.5 and 44 μg/ml in TMK-1, MKN-45, MKN-74 and CDDP-resistant MKN-74 cells, respectively. For 5-FU, IC_{50} values were determined as 27.1, 70.6, and 63.2 μg/ml in TMK-1, MKN-45 and MKN-74, respectively. These IC_{50} values were applied to the final drug concentration of each experiment.

Time-dependent alteration of gene expression in TMK-1 (Table II and Fig. 1). For TMK-1 cells, of the 96 genes on the array, 65 genes in the CDDP treatment group and 63 genes in the 5-FU treatment group were successfully quantified before and after drug exposure. The expression of 7 genes was significantly up-regulated at 8 h after CDDP treatment, and the number of up-regulated genes increased to 41 at 24 h after CDDP exposure. The expression of 3 genes was down-regulated 8 and 24 h after CDDP exposure. For 5-FU treatment, no genes were found to be up-regulated at 8 h, but the

Table I. The genes investigated.

Gene name	Assay ID	Ref. sequence no.	Description
GAPD	Hs99999905_m1	NM_002046	Glyceraldehyde-3-phosphate dehydrogenas
ACTB	Hs99999903_m1	NM_001101	Actin, ß
ITGB6	Hs00168458_m1	NM_000888	Integrin, ß6
MUC6	Hs00401231_m1	U97698	Mucin 6, gastric
ID4	Hs00155465_m1	NM_001546	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
TLE4	Hs00419101_m1	NM_007005	Transducin-like enhancer of split 4 (E(sp1)homolog, *Drosophila*)
	Hs00399505_m1		
RIPK3	Hs00179132_m1	NM_006871	Receptor-interacting serine-threonine kinase 3
IGF2	Hs00171254_m1	NM_000612	Insulin-like growth factor 2 (somatomedin A
TGFB2	Hs00236092_m1	NM_003238	Transforming growth factor, ß2
	Hs00234244_m1		
THBS4	Hs00170261_m1	NM_003248	Thrombospondin 4
CDCA7	Hs00230589_m1	NM_031942	Cell division cycle associated 7
		NM_145810	
RRM2B	Hs00394046_m1	NM_015713	Ribonucleotide reductase M2 B (TP53 inducible)
	Hs00153085_m1	NM_015713	
ITGAX	Hs00174217_m1	NM_000887	Integrin, αX (antigen CD11C (p150), α polypeptide)
LCP1	Hs00158701_m1	NM_002298	Lymphocyte cytosolic protein 1 (L-plastin)
TYMS	Hs00426591_m1	NM_001071	Thymidylate synthetase
DPYD	Hs00559278_m1	NM_000110	Dihydropyrimidine dehydrogenasz
UMPS	Hs00165978_m1	NM_000373	Uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)
ECGF1	Hs00157317_m1	NM_001953	Endothelial cell growth factor 1 (platelet-derived)
RRM1	Hs00168784_m1	NM_001033	Ribonucleotide reductase M1 polypeptide
RRM2	Hs00357247_g1	NM_001034	Ribonucleotide reductase M2 polypeptide
ERCC1	Hs00157415_m1	NM_001983	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)
UNG	Hs00422172_m1	NM_003362	Uracil-DNA glycosylas
		NM_080911	
ADPRT	Hs00242302_m1	NM_001618	ADP-ribosyltransferase [NAD+; poly (ADP-ribose)polymerase]
TOP1	Hs00243257_m1	NM_003286	Topoisomerase (DNA) I
ABCB1	Hs00184500_m1	NM_000927	ATP-binding cassette, sub-family B(MDR/TAP), member 1
	Hs00184491_m1	NM_000927	
SLC29A1	Hs00191940_m1	NM_004955	Solute carrier family 29 (nucleoside transporters), member 1
ABCC1	Hs00219905_m1	NM_004996	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
		NM_019898	
		NM_019901	
		NM_019902	
		NM_019899	
		NM_019862	
		NM_019900	
TOP2A	Hs00172214_m1	NM_001067	Topoisomerase (DNA) IIα 170 kDa
TOP2B	Hs00172259_m1	NM_001068	Topoisomerase (DNA) IIß 180 kDa
HSPB1	Hs00356629_g1	NM_001540	Heat shock 27 kDa protein 1
E2F1	Hs00153451_m1	NM_005225	E2F transcription factor 1
TP53	Hs00153340_m1	NM_000546	Tumor protein p53 (Li-Fraumeni syndrome)
ITGA3	Hs00233722_m1	NM_002204	Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor)
		NM_005501	

Table I. Continued.

Gene name	Assay ID	Ref. sequence no.	Description
PCNA	Hs00427214_g1	NM_002592 NM_182649	Proliferating cell nuclear antigen
CAV1	Hs00184697_m1	NM_001753	Caveolin 1, caveolae protein, 22 kDa
HIF1A	Hs00153153_m1	NM_001530	Hypoxia-inducible factor 1, α subunit (basic helix-loop helix transcription factor)
ATM	Hs00175892_m1	NM_000051	Ataxia telangiectasia mutated (includes complementation groups A, C and D)
CCNA2	Hs00153138_m1	NM_001237	Cyclin A2
CCNB1	Hs00259126_m1	NM_031966	Cyclin B1
CCND1	Hs00277039_m1	NM_053056	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)
CCNE1	Hs00233356_m1	M73812	Cyclin E1
CCNH	Hs00236923_m1	NM_001239	Cyclin H
CDC2	Hs00176469_m1	NM_001786	Cell division cycle 2, G1 to S and G2 to M
CDC25A	Hs00153168_m1	NM_001789	Cell division cycle 25A
CDK2	Hs00608082_m1	NM_001798	Cyclin-dependent kinase 2
CDK4	Hs00175935_m1	NM_000075	Cyclin-dependent kinase
CDK7	Hs00757090_g1	NM_001799	Cyclin-dependent kinase 7 (MO15 homolog, *Xenopus laevis*, cdk-activating kinase)
CDKN1A	Hs00355782_m1	U03106	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	Hs00153277_m1	NM_004064	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN2A	Hs00233365_m1	NM_000077	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CHEK1	Hs00176236_m1	NM_001274	CHK1 checkpoint homolog (S. pombe)
DDI2	Hs00260555_m1	NM_032341	DNA-damage inducible protein 2
DDIT3	Hs00358796_g1	S40706	DNA-damage-inducible transcript 3
GADD45A	Hs00169255_m1	NM_001924	Growth arrest and DNA-damage-inducible, α
GADD45B	Hs00169587_m1	NM_015675	Growth arrest and DNA-damage-inducible, ß
GADD45G	Hs00198672_m1	NM_006705	Growth arrest and DNA-damage-inducible, γ
PDCD8	Hs00269879_m1	NM_145812	Programmed cell death 8 (apoptosis-inducing factor)
PRKDC	Hs00179161_m1	NM_006904	Protein kinase, DNA-activated, catalytic polypeptide
YWHAG	Hs00705917_s1	NM_012479	Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, γ polypeptide
ABCA1	Hs00194045_m1	NM_005502	ATP-binding cassette, sub-family A (ABC1), member 1
ABCC2	Hs00166123_m1	NM_000392	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
ABCC5	Hs00194701_m1	NM_005688	ATP-binding cassette, sub-family C (CFTR/MRP), member 5
ABCG2	Hs00184979_m1	AB056867	ATP-binding cassette, sub-family G (WHITE), member 2
CDH1	Hs00170423_m1	NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)
CREB1	Hs00231713_m1	NM_004379	CAMP responsive element binding protein 1
CTNNB1	Hs00170025_m1	NM_001904	Catenin (cadherin-associated protein), ß1, 88 kDa
DNMT1	Hs00154749_m1	NM_001379	DNA methyltransferase 1
DNMT2	Hs00189402_m1	NM_176086	DNA methyltransferase 2
DNMT3A	Hs00601097_m1	NM_153759	DNA methyltransferase 3A
DNMT3B	Hs00171876_m1	NM_175849	DNA methyltransferase 3B
GSTP1	Hs00168310_m1	NM_000852	Glutathione S-transferase pi
GSR	Hs00167317_m1	M_000637	Glutathione redactas
HDAC4	Hs00195814_m1	NM_006037	Histone deacetylase 4
HDAC9	Hs00206843_m1	NM_002189	Histone deacetylase
IL15	Hs00542562_m1	NM_172174	Interleukin 15
IL15RA	Hs00233692_m1	NM_002189	Interleukin 15 receptor, α
IL2	Hs00174114_m1	NM_000586	Interleukin 2

Table I. Continued.

Gene name	Assay ID	Ref. sequence no.	Description
IL2RA	Hs00166229_m1	NM_000878	Interleukin 2 receptor, ß
IL2RG	Hs00173950_m1	NM_000206	Interleukin 2 receptor, γ
IL4	Hs00174122_m1	NM_002189	Interleukin 4
IL4R	Hs00166237_m1	NM_000418	Interleukin 4 receptor
IL7	Hs00174202_m1	NM_000880	Interleukin 7
IL7R	Hs00233682_m1	NM_002185	Interleukin 7 receptor
ITGB3	Hs00173978_m1	NM_000212	Integrin, ß3 (platelet glycoprotein IIIa, antigen CD61)
JUN	Hs00277190_s1	NM_002228	V-jun sarcoma virus 17 oncogene homolog (avian)
MYC	Hs00153408_m1	NM_002467	V-myc myelocytomatosis viral oncogene homolog (avian)
NOTCH1	Hs00413187_m1	NM_017617	Notch homolog 1, translocation-associated (*Drosophila*)
PTGS2	Hs00153133_m1	NM_000963	Prostaglandin-endoperoxide synthase 2
RB1	Hs00153108_m1	NM_000321	Retinoblastoma 1 (including osteosarcoma)
STAT1	Hs00234829_m1	NM_007315	Signal transducer and activator of transcription 1, 91 kDa
STAT1	Hs00234829_m1	NM_007315	Signal transducer and activator of transcription 1, 91 kDa
STAT3	Hs00234174_m1	NM_139276	Signal transducer and activator of transcription 3 (acute-phase response factor)
TUBB4	Hs00801390_s1	NM_006086	Tubulin, ß, 4
VEGF	Hs00173626_m1	NM_003376	Vascular endothelial growth factor
Wnt4	Hs00229142_m1	NM_030761	Wingless-type MMTV integration site family, member

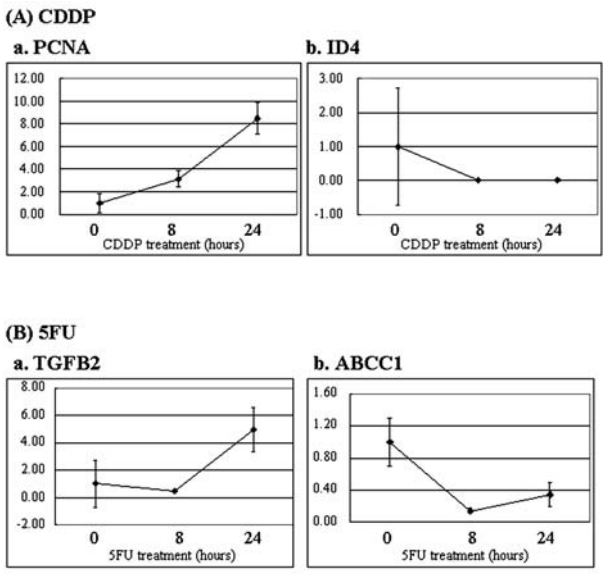

Figure 1. Represents genes of time-dependent alterations in TMK-1 after treatment by (A) CDDP and (B) 5-FU.

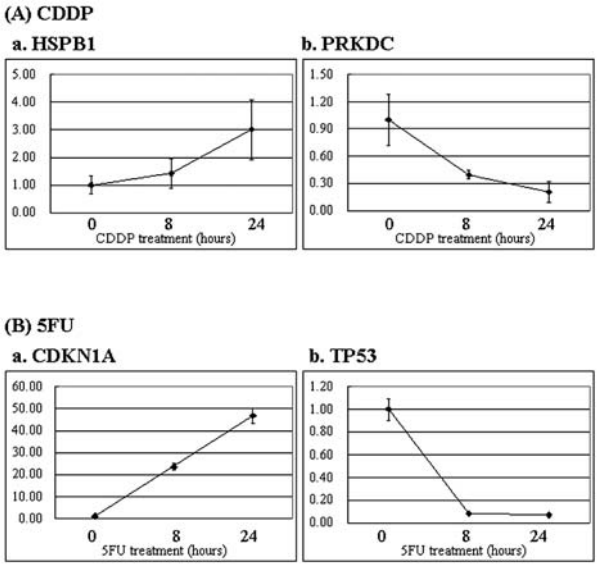

Figure 2. Represents genes of time-dependent alterations in MKN-45 after treatment by (A) CDDP and (B) 5-FU.

expression of 22 genes was up-regulated at 24 h after 5-FU exposure. The expression of 19 genes was down-regulated at 8 h after 5-FU exposure; among these 19 genes, 10 genes recovered to baseline (0 h), 6 genes were up-regulated and the remaining 3 genes continued to be down-regulated 24 h after 5-FU exposure.

Time-dependent alteration of gene expression in MKN-45 (Table III and Fig. 2). For MKN-45 cells, of the 96 genes on the array, 52 genes were successfully quantified throughout the experiment with CDDP and 5FU exposure. The expression of 3 genes was up-regulated at 8 h after CDDP exposure, and 2 of these 3 genes showed enhanced expression at 24 h, while the other gene recovered to the baseline level. The number of up-regulated genes increased from 3 genes at 8 h to 13 genes at 24 h after CDDP exposure. The expression of 6 genes was down-regulated at 8 h after CDDP treatment; of these 6 genes, 3 genes recovered to

Table II. Time-dependent alterations of gene expression in TMK-1.

a. CDDP	
Up-regulated	
After 8 h	UMPS, UNG, PCNA, CDC25A, YWHAG, CTNNB1, TUBB4
After 24 h	UMPS, RRM1, RRM2, ERCC1, UNG, ADPRT, TOP1, TOP2A, TOP2B, HSPB1, E2F1, TP53, PCNA, HIF1A, CCNB1, CCNE1, CCND1, CDC2, CDC25A, CDK2, CDK4, CDK7, CDKN1A, CDKN1B, DDI2,GADD45A, PDCD8, YWHAG, ABCC2, ABCG2, CREB1, CTNNB1, GSTP1, GSR, IL15RA, JUN, MYC,RB1, STAT1, STAT3, TUBB4
Down-regulated	
After 8 h	ID4, IL4, ITGB3
After 24 h	ID4, IL4, ITGB3
b. 5-FU	
Up-regulated	
After 8 h	None
After 24 h	ID4, TGFB2, RRM2B, UMPS, RRM1, RRM2, UNG, TOP1, SLC29A1, CCNE1, CDC2, CDK2, CDK4, CDKN1A, CHEK1, GADD45B, ITGB3, NOTCH1, PTGS2, RB1, STAT1, VEGF
Down-regulated	
After 8 h	UMPS, RRM1, RRM2, TOP2A, HSPB1, TP53, HIF1A, CCNB1, ABCC1, CDC2, CDK2, CDK7, ABCG2,CTNNB1, GSTP1, GSR, RB1, STAT3
After 24 h	ABCC1, PDCD8, CTNNB1

Table III. Time-dependent alterations of gene expression in MKN-45.

a. CDDP	
Up-regulated	
After 8 h	CDKN1A, GADD45A, GADD45G
After 24 h	RRM2B, HSPB1, TP53, ITGA3, PCNA, CCNE1, CDKN1A, CDKN1B, GADD45A, GADD45B, YWHAG, CTNNB1, TUBB4
Down-regulated	
After 8 h	TOP1, SLC29A1, CDC2, PRKDC, ABCG2, MYC
After 24 h	PRKDC, ABCG2, CDH1, MYC
b. 5-FU	
Up-regulated	
After 8 h	ID4, CDKN1A, GADD45B, ABCC5
After 24 h	ID4, ATM, CDC25A, CDKN1A, GADD45G, ABCC5, VEGF
Down-regulated	
After 8 h	UMPS, RRM2, ERCC1, UNG, TOP1, TOP2B, HSPB1, TP53, HIF1A, CCNB1, CCNH, CDC2, CDK2, PDCD8, PRKDC, ABCG2, CREB1, CTNNB1, GSR, MYC
After 24 h	RRM2B, ERCC1, UNG, TOP1, TOP2B, TP53, HIF1A, CCNB1, CCNH, CDC2, CDK2, CDKN1B, PDCD8, PRKDC, YWHAG, ABCG2, CDH1, CREB1, CTNNB1, GSR, MYC

baseline and the other 3 genes remained down-regulated at 24 h. For 5-FU treatment, the expression of 4 genes was up-regulated at 8 h after 5-FU exposure; at 24 h post-treatment, 1 of these genes recovered to baseline. The number of up-regulated genes increased to 7 at 24 h. Twenty genes were down-regulated at 8 h, and 3 out of these 20 genes recovered to baseline levels at 24 h. The number of down-regulated genes increased to 21 at 24 h

Time-dependent alteration of gene expression in MKN-74 (Table IV and Fig. 3). For MKN-74 cells, of the 96 genes, 40 genes in the CDDP treatment group and 41 genes in the 5-FU

Table IV. Time-dependent alterations of gene expression in MKN-74.

a. CDDP	
Up-regulated	
After 8 h	ERCC1, HSPB1, TP53, CDKN1A, DDIT3, GADD45A, ABCG2, CTNNB1, JUN
After 24 h	ID4, IGF2, RRM2B, RRM2, ERCC1, HSPB1, TP53, CDK4, CDKN1A, CDKN1B,CDKN2A, DDIT3, GADD45A, GADD45B, GADD45G, YWHAG, ABCG2, CDH1, CREB1, CTNNB1, JUN, NOTCH1, RB1, STAT3
Down-regulated	
After 8 h	TYMS, UMPS, UNG, TOP1, TOP2B, CCND1, DNMT1, GSTP1, GSR, MYC, STAT1
After 24 h	TYMS, UMPS, DNMT1
b. 5-FU	
Up-regulated	
After 8 h	IGF2, RRM2B, HSPB1, CDKN1B, CDH1, CREB1, PTGS2
After 24 h	IGF2, RRM2B, UMPS, RRM1, UNG, TOP1, SLC29A1, TOP2B, HSPB1, HIF1A, CDKN1B, CDKN2A, CHEK1, GADD45B, CREB1, MYC, PTGS2
Down-regulated	
After 8 h	ID4, TYMS, UMPS, RRM1, RRM2, UNG, CCND1, CDK4, CHEK1, GADD45A GADD45G, YWHAG, CTNNB1, DNMT1, GSR, JUN, NOTCH1, RB1, STAT1, STAT3
After 24 h	GADD45A, GADD45G, DNMT1, GSR, JUN, STAT1,

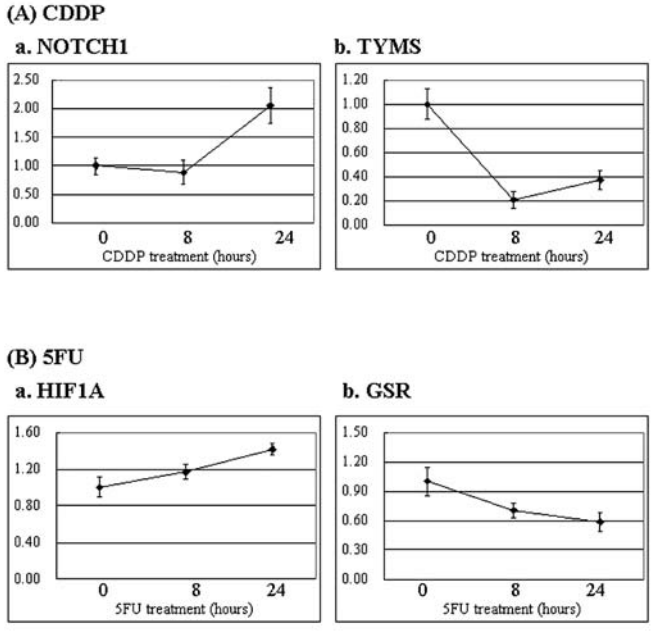

Figure 3. Represents genes of time-dependent alterations in MKN-74 after treatment by (A) CDDP and (B) 5-FU.

treatment group were successfully quantified. Nine genes were up-regulated at 8 h after CDDP exposure, and 24 genes were up-regulated at 24 h after CDDP exposure. Eleven genes were down-regulated at 8 h after CDDP exposure, and this number decreased to 3 genes at 24 h after CDDP exposure. For 5-FU treatment, 7 genes were up-regulated at 8 h, and 6 of these 7 genes showed enhanced up-regulation at 24 h after 5-FU exposure; the seventh returned to baseline at 24 h after 5-FU exposure. A total of 17 genes were up-regulated at 24 h after 5-FU exposure. On the other hand, 20 genes were down-regulated at 8 h after 5-FU exposure; out of these 20 genes, the expression of 10 genes recovered to baseline, while 6 genes remained down-regulated and 4 genes showed up-regulation (compared to baseline levels) at 24 h after 5-FU exposure.

Table V. Gene analysis of CDDP-resistant MKN-74 relative to MKN-74 at 0 h.

Increase in expression
ID4, HSPB1, TOP2B, E2F1, PCNA, ATM, CCNA2, CCNB1, CCNH, CDC2, CDC25A, CDK2, CDK7, CDKN2A, PDCD8, PRKDC, DNMT1, GSTP1, IL4, PTGS2, UBB4
Decrease in expression
IGF2, RRM2B, TYMS, UMPS, RRM1, RRM2, TP53, HIF1A, CCND1, CDK4, CDKN1B, GADD45A, GADD45B, GADD45G, YWHAG, ABCG2, CREB1, CTNNB1, GSR, JUN, NOTCH1, RB1, STAT1, STAT3

Gene analysis of CDDP-resistant MKN-74 (Tables V and VI; Figs. 4 and 5). CDDP-resistant MKN-74 cells without drug exposure showed up-regulation in 21 genes and down-regulation in 24 genes compared with the parental MKN-74 cell line (Table V).

Of the 96 genes on the array, 44 genes were successfully evaluated after CDDP exposure. The substantial differences in gene alteration by CDDP were confirmed between the

Table VI. Time-dependent alterations of gene expression in CDDP-resistant MKN-74.

Up-regulated	
After 8 h	TP53, JUN
After 24 h	TP53, JUN
Down-regulated	
After 8 h	ID4, IGF2, RRM1, UNG,TOP2B, E2F1, PCNA, HIF1A, ATM, CCNB1, CDC2, CDC25A, CDK2, CDK4, CDK7, CDKN1B, CDKN2A, GADD45B, PDCD8, PRKDC, ABCG2, CDH1, DNMT1, GSTP1, GSR, IL4, MYC
After 24 h	ID4, IGF2, RRM1, RRM2, UNG, TOP1, TOP2B, E2F1, PCNA, HIF1A, ATM, CCNA2, CCNB1, CDC2, CDC25A, CDK2, CDK4, CDK7, CDKN1B, GADD45B, PDCD8, PRKDC, ABCG2, CDH1, DNMT1, GSTP1, GSR, IL4, MYC, PTGS2, STAT1

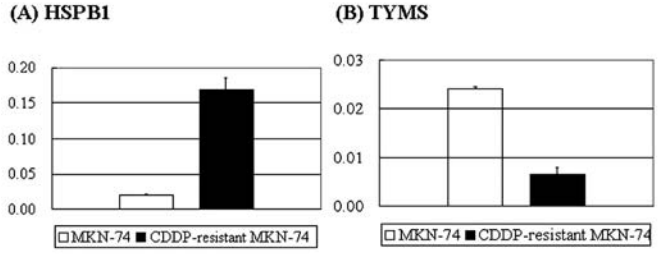

Figure 4. Represents genes that showed up-regulation and down-regulation in CDDP-resistant MKN-74 compared with the parental cell line without drug exposure.

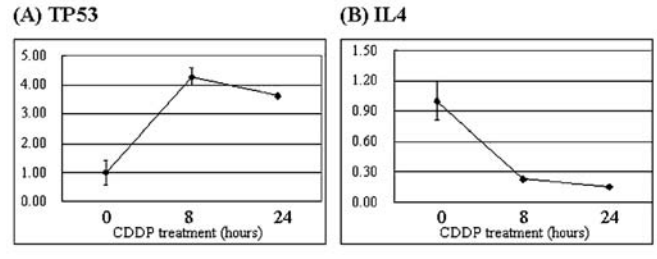

Figure 5. Represents genes of time-dependent alteration.

parental MKN-74 and the CDDP-resistant MKN-74 cells. Interestingly, the up-regulation of genes by CDDP exposure was reduced (only 2 genes vs. 24 genes in the parental cell line at 24 h after exposure. On the other hand, of the 44 genes, 28 genes were down-regulated at 8 h after the treatment; this number increased to 31 genes at 24 h after the treatment. Of the 28 genes with decreased expression at 8 h, only 1 gene recovered to baseline at 24 h.

Discussion

This study has demonstrated that there is a wide range of gene expression profiles in human gastric cancer cell lines in response to stimulation by the most commonly used chemotherapeutic drugs, CDDP and 5-FU, in the clinic. Such diverse gene expression profiles in cell lines derived from different people, who possibly had different chemotherapeutic outcomes, demonstrate the underlying biological complexity that is a major determinant in therapeutic outcome. We also established CDDP-resistant MKN-74 cells and compared gene expression with and without drug exposure to the parental MKN-74 cell line. Interestingly, acquired CDDP-resistant MKN-74 cells did not show the same gene expression pattern as the parental MKN-74 at 0, 8 and 24 h after CDDP exposure. A comparison of gene expression between the two cell lines at 0 h did show various kinds of alterations associated with CDDP resistance, strongly suggesting that baseline gene expression before drug exposure will not always predict gene expression alteration after drug exposure. Only 2 genes showed up-regulation in the CDDP-resistant MKN-74 cells, and this resistance to gene up-regulation may be one significant predictor for chemoresistance throughout chemotherapy. In contrast, 31/41 (75.6%) genes were down-regulated in the CDDP-resistant MKN-74 cells after CDDP exposure.

Several reports have suggested that the mechanisms of resistance against CDDP are not completely understood and seem to involve many factors (21,22). Modifications of CDDP metabolism inside cells, drug transportation, enhanced intracellular detoxification or increased repair and/or tolerance to DNA damage are possible mechanisms of CDDP resistance with various molecular changes (23-26). However, such mechanisms were only discussed relating to acquired-resistance cells; and in the field of chemosensitivity/chemoresistance in cancer cells, dynamic alterations of genes at the mRNA level have not yet been in focus. This study has uniquely shown various kinds of gene alteration after chemotherapy among different gastric cancer cell lines and between acquired CDDP-resistant cell lines and the parental cell line. This suggests that complex interactions involving many genes and genetic and epigenetic phenotypes occur after anticancer drug exposure. However, some genes with altered expression by CDDP or 5-FU are not necessarily directly related to sensitivity or resistance to CDDP; thus, some of the gene alterations evaluated with the present array include incidental alteration (27).

Recent trials to predict chemosensitivity using analysis of chemosensitivity/chemoresistance-related genes, such as thymidine synthase, dihydropyrimidine dehydrogenase or ATP binding cassette family, are reported to have good potential for clinical use (28-30); however, our demonstration of the dynamic alteration of gene expression after anticancer drug exposure will completely deny the role of prediction of chemotherapeutic outcome by a one-point measurement of baseline gene expression, especially without a specific therapy. Furthermore, this study suggests that, even among gastric cancer patients, various gene dynamics will occur, suggesting clinical difficulties in predicting chemotherapeutic outcome by measuring dynamic gene expression

alteration even in one kind of cancer cell after one kind of chemotherapy. Interestingly, recent work on gene dynamics in fetal development, immunity, and the stem cell field has shown similar results. This recent study suggests that cancer cells themselves are adapting to the altered microenvironment induced by anticancer drugs (31-34). Ultimately, prediction of chemotherapeutic outcome in clinics should include baseline values and dynamic values of complex gene expression with drug exposure, as well as host dynamics such as drug distribution, metabolism, and toxicity with drugs. In this study, the focus on the *in vitro* human cancer cell line system, neglecting host factors, highlights the biological significance of understanding gene expression dynamics especially in cancer cells, as well as the inherent biological nature of cancer cells. This study may provide a foundation for the development of new treatments or experimental protocols, focusing on the complexity of biological and pathological events, to achieve a desirable outcome of chemotherapy.

References

1. Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24: 2137-2150, 2006.
2. Sakuramoto S, Sasako M, Yamaguchi T, *et al*: Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N Engl J Med 357: 1810-1820, 2007.
3. Ohtsu A: Current status and future prospects of chemotherapy for metastatic gastric cancer: a review. Gastric Cancer 8: 95-102, 2005.
4. Schipper DL and Wagener DJT: Chemotherapy of gastric cancer. Anticancer drugs 7: 137-149, 1995.
5. Hu JK, Chen ZX, Zhou ZG, *et al*: Intravenous chemotherapy for resected gastric cancer. Meta-analysis of randomized controlled trials. World J Gastroenterol 8: 1023-1028, 2002.
6. GolubTR, Slonim DK, Tamayo P, *et al*: Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286: 531-537, 1999.
7. Ulrich CM, Robien K and McLeod HL: Cancer pharmacogenetics: polymorphisms, pathways and beyond. Nat Rev Cancer 12: 912-920, 2003.
8. Banerjee D, Mayer-Kuckuk P, Capiaux G, *et al*: Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. Biochim Biophys Acta 1587: 164-173, 2002.
9. Gottesman MM: Mechanisms of cancer drug resistance. Annu Rev Med 53: 615-627, 2002.
10. Yatomi M, Takiguchi Y, Asaka-Amano Y, *et al*: Altered gene expression by Cisplatin in a human squamous cell lung carcinoma cell line. Anticancer Res 27: 3235-3244, 2007.
11. Park JS, Yoon SY, Kim JM, *et al*: Identification of novel genes associated with the response to 5-FU treatment in gastric cancer cell lines using a cDNA microarray. Cancer Lett 214: 19-33, 2004.
12. Yuan R, Fan S, Achary M, *et al*: Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/Scatter factor in the setting of DNA damage. Cancer Res 61: 8022-8031, 2001.
13. Wong N, Chan KY, Macgregor PF, *et al*: Transcriptional profiling identifies gene expression changes associated with IFN-α tolerance in hepatitis C-related hepatocellular carcinoma cells. Clin Cancer Res 11: 1319-1325, 2005.
14. Ranganathan P, Agrawal A, Bhushan R, *et al*: Expression profiling of genes regulated by TGF-beta: Differential regulation in normal and tumor cells. BMC Genomics 8: 98, 2007.
15. Rafferty M, Faller WJ, Moss C, *et al*: Real-time quantitative reverse transcriptase-polymerase chain reaction analysis of melanoma progression-associated genes. Anticancer Res 27: 1301-1307, 2007.
16. Lyng MB, Laenkholm AV, Pallisgaard N, *et al*: Intratumor genetic heterogeneity of breast carcinomas as determined by fine needle aspiration and TaqMan low density array. Cell Oncol 29: 361-372, 2007.
17. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.
18. Shimoyama Y, Kubota T, Watanabe M, *et al*: Predictability of *in vivo* chemosensitivity by *in vitro* MTT assay with reference to the clonogenic assay. J Surg Oncol 41: 12-18, 1989.
19. Suto A, Kubota T, Shimoyama Y, *et al*: MTT assay with reference to the clinical effect of chemotherapy. J Surg Oncol 42: 28-32, 1989.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 25: 402-408, 2001.
21. Andrews PA: Mechanisms of acquired resistance to cisplatin. Cancer Treat Res 73: 217-248, 1994.
22. Gosland M, Lum B, Schimmelpfennig J, *et al*: Insights into mechanisms of cisplatin resistance and potential for its clinical reversal. Pharmacotherapy 16: 16-39, 1996.
23. Mann SC, Andrews PA and Howell SB: Comparison of lipid content, surface membrane fluidity, and temperature dependence of cis-diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Anticancer Res 8: 1211-1215, 1988.
24. Kubbies M, Goller B, Schetters B, *et al*: Glutathione restores normal cell activation and cell cycle progression in cis-platinum treated human lymphocytes. Br J Cancer 64: 843-849, 1991.
25. Kasahara K, Fujiwara Y, Nishio K, *et al*: Metallothionein content correlates with sensitivity of human small cell lung cancer cell lines to cisplatin. Cancer Res 51: 3237-3242, 1991.
26. Masuda H, Tanaka T, Matsuda H, *et al*: Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatinum (II). Cancer Res 50: 1863-1866, 1990.
27. Macgregor PF and Squire JA: Application of microarrays to the analysis of gene expression in cancer. Clin Chem 48: 1170-1177, 2002.
28. Aschele C, Lonardi S and Monfardini S: Thymidylate synthase expression as a predictor of clinical response to fluoropyrimidine-based chemotherapy in advanced colorectal cancer. Cancer Treat Rev 28: 27-47, 2002.
29. Isshi K, Sakuyama T, Gen T, *et al*: Predicting 5-FU sensitivity using human colorectal cancer specimens: comparison of tumor dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase activities with in vitro chemosensitivity to 5-FU. Int J Clin Oncol 7: 335-342, 2002.
30. Scherf U, Ross DT, Waltham M, *et al*: A gene expression database for the molecular pharmacology of cancer. Nat Genet 24: 236-244, 2000.
31. Ieta K, Tanaka F, Haraguchi N, *et al*: Biological and genetic characteristics of tumor-initiating cells in colon cancer. Ann Surg Oncol 15: 638-648, 2008.
32. Zhou J, Wulfkuhle J, Zhang H, *et al*: Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci USA 104: 16158-16163, 2007.
33. Haraguchi N, Utsunomiya T, Inoue H, *et al*: Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 24: 506-513, 2006.
34. Tsuneyoshi N, Sumi T, Onda H, *et al*: PRDM14 suppresses expression of differentiation marker genes in human embryonic stem cells: Biochem Biophys Res Commun 367: 899-905, 2008.