Alteration of gene expression and DNA methylation in drug-resistant gastric cancer

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Abstract. The mechanisms of drug resistance in cancer are not fully elucidated. To study the drug resistance of gastric cancer, we analyzed gene expression and DNA methylation profiles of 5-fluorouracil (5-FU)- and cisplatin (CDDP)-resistant gastric cancer cells and biopsy specimens. Drug-resistant gastric cancer cells were established with culture for >10 months in a medium containing 5-FU or CDDP. Endoscopic biopsy specimens were obtained from gastric cancer patients who underwent chemotherapy with oral fluoropyrimidine S-1 and CDDP. Gene expression and DNA methylation analyses were performed using microarray, and validated using real-time PCR and pyrosequencing, respectively. Out of 17,933 genes, 541 genes commonly increased and 569 genes decreased in both 5-FU- and CDDP-resistant AGS cells. Genes with expression changed by drugs were related to GO term 'extracellular region' and 'p53 signaling pathway' in both 5-FU- and CDDP-treated cells. Expression of 15 genes including KLK13 increased and 12 genes including ETV7 decreased, in both drug-resistant cells and biopsy specimens of two patients after chemotherapy. Out of 10,365 genes evaluated with both expression microarray and methylation microarray, 74 genes were hypermethylated and downregulated, or hypomethylated and upregulated in either 5-FU-resistant or CDDP-resistant cells. Of these genes, expression of 21 genes including FSCN1, CPT1C and NOTCH3, increased from treatment with a demethylating agent. There are alterations of gene expression and DNA methylation in drug-resistant gastric cancer; they may be related to mechanisms of drug resistance and may be useful as biomarkers of gastric cancer drug sensitivity.

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Key words: gastric cancer, cisplatin, 5-fluorouracil, drug resistance, DNA methylation

Introduction

Gastric cancer is one of the most common causes of cancer-related mortality, responsible for >700,000 deaths worldwide per year (1). Although the main treatment strategy for gastric cancer is surgical or endoscopic resection, unresectable cases are treated with systemic chemotherapy. Platinum agents and fluoropyrimidine are the key therapeutic drugs for advanced gastric cancer (2) and drug resistance is an important problem accompanying treatment. A number of studies have previously reported on the mechanisms of gastric cancer chemoresistance (3) using cultured cells, animal models and clinical tissue samples. However, the mechanisms of drug resistance have not been fully elucidated.

It has been reported that both genetic and epigenetic changes play important roles in carcinogenesis and tumor progression (4). In various types of cancer, epigenetic changes are known to be early events in the multi-steps of carcinogenesis (5). Promoter hypermethylation is well-known to be important for the suppression of tumor suppressor gene expression (4). Mechanisms of cancer drug resistance are considered to be multifactorial; they have epigenetic alterations (6) and involve multiple gene functions and signaling pathways. A better understanding of such mechanisms may provide therapeutic strategies for gastric cancer.

In the present study, drug-resistant gastric cancer cell lines were established, and biopsy specimens were obtained from patients after the acquisition of drug resistance. Genome-wide analysis of gene expression and DNA methylation with a microarray for drug-resistant cell lines and endoscopic biopsy specimens of gastric cancer was performed. Validation with quantitative methods was also performed.

Materials and methods

Cell culture and 5-aza-2'-deoxycytidine (5-aza-dC) treatment. AGS was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and cultured in RPMI-1640 medium with 10% FBS at 37˚C with 5% CO_2. For treatment with 5-aza-dC (decitabine), cells were seeded on day 0, and exposed to freshly prepared 10 μmol/l 5-aza-dC (Sigma-Aldrich, Tokyo, Japan) for 24 h on days 1 and 3. After

each treatment, the cells were placed in fresh medium and harvested on day 4 (7).

Drug-resistant gastric cancer cells. Resistant AGS cells were generated by continuous exposure to increasing concentrations of cisplatin (CDDP) or 5-fluorouracil (5-FU) for 10 months. Viability of cells was measured by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). AGS cells ($2x10^3$) were cultured using 96-well microplates for 24 h, and exposed to various concentrations of CDDP or 5-FU for 48 h to calculate the IC_{50} of CDDP or 5-FU for each cell line.

Patients and biopsy specimens. Endoscopic biopsy specimens were obtained from two patients with unresectable advanced gastric cancer who underwent 3 and 4 courses of chemotherapy with oral fluoropyrimidine S-1 plus CDDP (8) before and after treatment. Samples after chemotherapy were obtained from lesions with viable cancer. The present study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine, and written informed consent was provided by the patients.

Extraction of DNA and RNA from cell lines and gastric biopsy specimens. Cells or biopsy specimens were stored at -80˚C for DNA extraction, and we used RNAlater (Ambion, Austin, TX, USA) for RNA extraction. For extraction of DNA and RNA, DNA Mini kit (Qiagen, Venlo, The Netherlands) and RNA Mini kit (Qiagen) were used, respectively.

Gene expression analysis with microarray. Expression analysis was performed with SurePrint G3 Human GE 8x60K (Agilent, Loveland, CO, USA). Expression of mRNA of autosomal 17,933 genes was evaluated with 23,856 corresponding probes. A difference in signal intensity >2-fold was judged to be significant.

DNA methylation microarray. Bisulfite-converted DNA was used for hybridization on Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA). The β-value [intensity of the methylated allele (M)/(intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100)] was calculated for each CpG site (9). Methylation levels of candidate promoter lesions in CpG islands of 11,692 genes were evaluated. Genes with a difference in their β-value >0.1 were extracted. Of these 11,692 genes, expression of 10,365 genes was also evaluated with expression microarray.

Gene ontology (GO) and pathway analysis. GO analysis was performed with TargetMine (http://targetmine.nibio.go.jp/targetmine/begin.do; National Institute of Biomedical Innovation, Osaka, Japan), and pathway analysis was performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. GO terms or pathways with $P<0.05$ using the Holm-Bonferroni method were judged to be significantly enriched.

Real-time PCR. Real-time PCR was performed to validate expression of mRNA with TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (both from Applied Biosystems, Foster City, CA, USA).

Bisulfite pyrosequencing. Bisulfite treatment was performed with the EpiTect kit (Qiagen) according to the manufacturer's protocol. PyroMark PCR (Qiagen) was used to perform PCR, and bisulfite pyrosequencing was performed as previously reported (10-12). In brief, the biotinylated PCR product was captured on streptavidin-coated beads (Amersham Biosciences, Uppsala, Sweden) and run on the PSQHS Pyrosequencing System (Biotage, Uppsala, Sweden) to obtain the degree of methylation.

Results

Resistance to CDDP or 5-FU of established drug-resistant cell lines. To confirm whether gastric cancer cells cultured in chemotherapy agents obtained drug resistance, IC_{50} values were measured. IC_{50} values of 5-FU were 10 and 56 μM in parent AGS and 5-FU-resistant AGS (5FUr), respectively. IC_{50} values of CDDP were 13 and 25 μM in parent AGS and CDDP-resistant AGS (CDDPr), respectively.

Genes with altered expression in drug-resistant cells and biopsy specimens. To characterize gene expression profiles of drug-resistant gastric cancer cells, expression microarray analysis was performed. A comparison of parent AGS, 5FUr and CDDPr change of expression is shown in Fig. 1. The expression of 541 genes increased and the expression of 569 genes decreased in both 5FUr and CDDPr compared with parent AGS. In contrast, the expression of only 25 genes increased in 5FUr and decreased in CDDPr, and the expression of only seven genes decreased in 5FUr and increased in CDDPr. To examine the characterization of genes with expression altered by drug treatment, we performed GO analysis and pathway analysis (Table I). Although most enriched GO terms differed between those changed in 5FUr and those changed in CDDPr, 'extracellular region' in GO terms of cellular component was commonly enriched in both 5FUr and CDDPr. With pathway analysis, the 'p53 signaling pathway' was enriched in both 5FUr and CDDPr.

To compare gene expression of gastric cancer before and after chemotherapy, expression microarray analysis with endoscopic biopsy specimens was performed. Genes with altered expression both in drug-resistant cells and in biopsy specimens after chemotherapy were extracted. The expression of 15 genes increased 5FUr, CDDPr and two pairs of biopsy specimens, and the expression of 12 genes decreased (Fig. 2, Table II).

To validate the gene expression change extracted with microarray, real-time PCR for KLK13 and ETV7 was performed. Consistent with microarray analysis, KLK13 increased in both drug-resistant AGS cells and endoscopic biopsy specimens after chemotherapy (Fig. 3A). In contrast, ETV7 decreased in both drug-resistant cells and biopsy specimens after treatment (Fig. 3B).

Integrated analysis of expression and methylation microarray. To study whether DNA methylation contributes to gene expression change caused by chemotherapy agents, we analyzed the methylation profiles of 5FUr and CDDPr and compared them with parent AGS. The number of genes that were hypermethylated and decreased in expression and that were hypomethylated and increased in expression was 74.

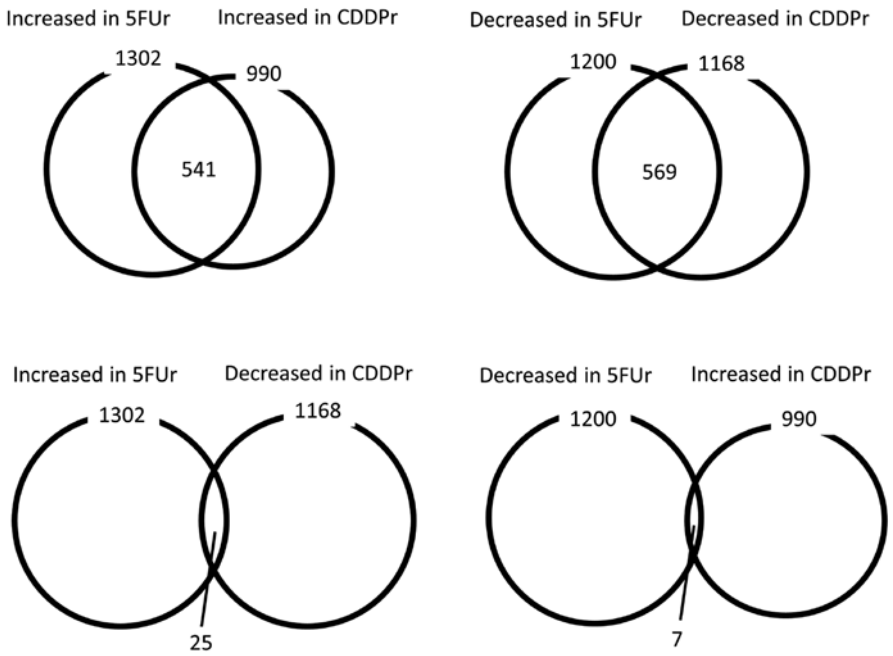

Figure 1. Venn diagrams showing number of genes in which expression increased or decreased with 5-FU or CDDP treatment. 5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

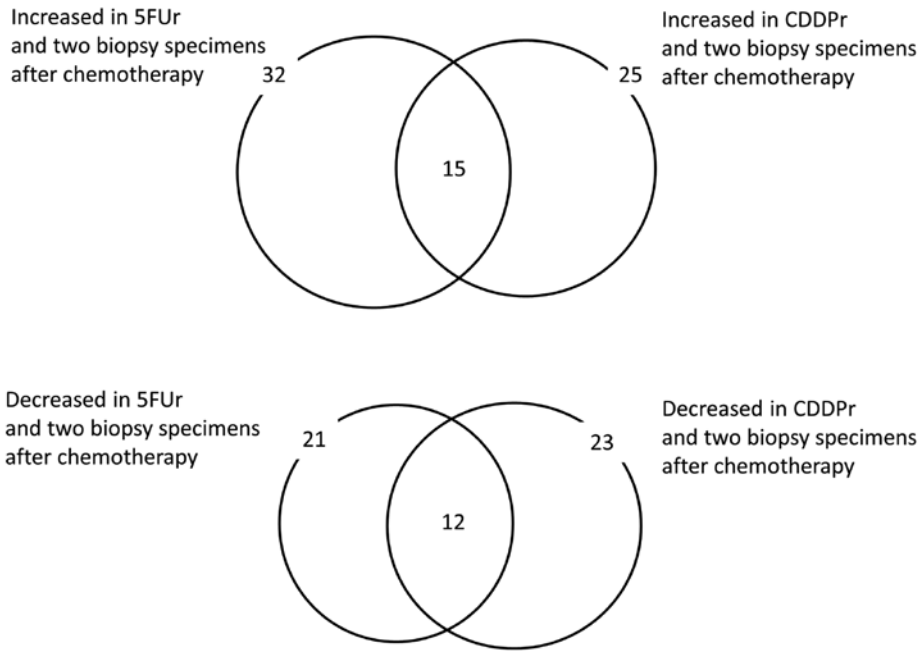

Figure 2. Venn diagrams showing number of genes in which expression was commonly changed in drug-resistant cells and biopsy specimens. 5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

Next, to evaluate whether alterations in the DNA methylation of these genes was related to expression change, gene expression change was measured by treatment with a demethylating agent. Twenty-one of those 74 genes increased in expression after treatment with decitabine (Table III). Furthermore, gene expression and methylation were validated with quantitative methods, TaqMan PCR and bisulfite pyrosequencing, respectively, for FSCN1, CPT1C and NOTCH3. Expression of these three genes increased after treatment with decitabine (Fig. 4). FSCN1 revealed increased expression and hypomethylation in CDDPr compared with parent AGS cells. Regarding CPT1C, 5FUr showed hypomethylation and increased expression. CDDPr also showed increased expression, although the methylation level did not change. NOTCH3 showed increased expression and hypomethylation, especially in 5FUr.

Table I. Significantly enriched gene ontology (GO) terms and pathways of genes in which expression was changed in 5-fluorouracil-resistant AGS cells (5FUr) and cisplatin-resistant AGS cells (CDDPr), compared with parent AGS cells.

A, GO terms (biological processes)

	GO terms (biological processes)		P-value	No. of genes
Increased in 5FUr	(No enrichment)			
Increased in CDDPr	Response to other organism	(GO:0051707)	0.000780	45
	Cell surface receptor signaling pathway	(GO:0007166)	0.00125	163
	Response to virus	(GO:0009615)	0.00180	31
	Response to biotic stimulus	(GO:0009607)	0.00288	45
	Signal transduction	(GO:0007165)	0.00382	247
	Regulation of signal transduction	(GO:0009966)	0.00511	125
	Positive regulation of cell communication	(GO:0010647)	0.00708	69
	Positive regulation of signaling	(GO:0023056)	0.00708	69
	Immune system process	(GO:0002376)	0.00867	130
	Regulation of cell motility	(GO:2000145)	0.0105	41
	Positive regulation of signal transduction	(GO:0009967)	0.0123	67
	Regulation of cell migration	(GO:0030334)	0.0146	39
	Single-organism process	(GO:0044699)	0.0151	516
	Regulation of signaling	(GO:0023051)	0.0159	132
	Regulation of cell communication	(GO:0010646)	0.0182	132
	Response to stimulus	(GO:0050896)	0.0206	341
	Regulation of response to stimulus	(GO:0048583)	0.0212	157
	Positive regulation of cell migration	(GO:0030335)	0.0224	27
	Regulation of cellular component movement	(GO:0051270)	0.0244	43
	Positive regulation of cell motility	(GO:2000147)	0.0308	27
	Regulation of localization	(GO:0032879)	0.0324	92
	Regulation of locomotion	(GO:0040012)	0.0496	41
Decreased in 5FUr	Defense response	(GO:0006952)	2.86E-05	110
	Immune system process	(GO:0002376)	0.000327	148
	Innate immune response	(GO:0045087)	0.000364	80
	Single-multicellular organism process	(GO:0044707)	0.000918	290
	Immune response	(GO:0006955)	0.0131	100
	Multicellular organismal process	(GO:0032501)	0.0161	295
Decreased in CDDPr	Single-multicellular organism process	(GO:0044707)	0.0343	279
	Xenobiotic catabolic process	(GO:0042178)	0.0478	5

B, GO terms (cellular components)

	GO terms (cellular components)		P-value	No. of genes
Increased in 5FUr	Extracellular region	(GO:0005576)	4.64E-07	130
	Cell periphery	(GO:0071944)	0.000754	280
	Plasma membrane	(GO:0005886)	0.000907	277
	Extracellular region part	(GO:0044421)	0.00647	70
	Plasma membrane part	(GO:0044459)	0.0134	136
	Extracellular space	(GO:0005615)	0.0271	56
Increased in CDDPr	Extracellular region	(GO:0005576)	0.0374	92
Decreased in 5FUr	Extracellular region	(GO:0005576)	3.24E-05	116
Decreased in CDDPr	Extracellular region	(GO:0005576)	7.37E-08	121
	Cornified envelope	(GO:0001533)	0.0124	8
	Intrinsic to membrane	(GO:0031224)	0.0175	142
	Integral to membrane	(GO:0016021)	0.0195	138
	Cell periphery	(GO:0071944)	0.0202	243
	Plasma membrane	(GO:0005886)	0.0268	240

Table I. Continued.

C, GO terms (molecular functions)

	GO terms (molecular functions)		P-value	No. of genes
Increased in 5FUr	Serine-type peptidase activity	(GO:0008236)	3.29E-05	24
	Serine hydrolase activity	(GO:0017171)	5.16E-05	24
	Serine-type endopeptidase activity	(GO:0004252)	0.00325	17
Increased in CDDPr	(No enrichment)			
Decreased in 5FUr	(No enrichment)			
Decreased in CDDPr	Receptor binding	(GO:0005102)	5.08E-04	83
	Cytokine activity	(GO:0005125)	0.0383	15

D, pathway

	Pathway	P-value	No. of genes
Increased in 5FUr	p53 signaling pathway	0.0222	14
Increased in CDDPr	p53 signaling pathway	8.56E-07	18
Decreased in 5FUr	(No enrichment)		
Decreased in CDDPr	Cytokine-cytokine receptor interaction	0.00618	37

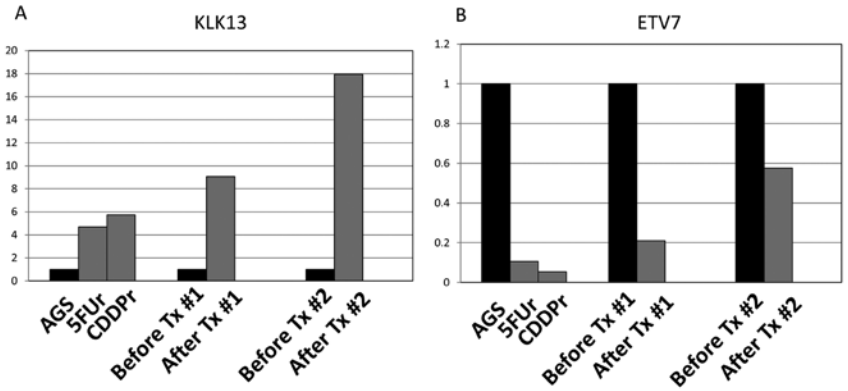

Figure 3. Relative expression levels in (A) KLK13 and (B) ETV7 in drug-resistant cells and biopsy specimens. Relative levels of parent AGS and biopsy specimens before treatment were set to one. Tx, chemotherapy; 5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

Discussion

The gene expression and the DNA methylation of drug-resistant cell lines and biopsy specimens were evaluated before and after chemotherapy, and some genes revealed altered expression and altered methylation. In drug-resistant cells, treatment with 5-FU and CDDP caused consistent expression change in >1,000 genes. In contrast, the expression of only a small number of genes changed reciprocally (Fig. 1), and those genes were considered to be potentially related with drug-specific sensitivity. In GO analysis, only a small number of GO terms are commonly enriched in both 5-FU-resistant cells and CDDP-resistant cells, and enriched pathways related to the two drugs are also different. These findings may be related to differences in mechanisms of resistance to each drug.

Expression change of genes before and after chemotherapy was also evaluated using endoscopic biopsy specimens, and revealed that profiles of changes were different in the two patients. Expression of some genes increased or decreased, both in drug-resistant cells and biopsy specimens after chemotherapy (Fig. 2, Table II). Such genes are considered to be candidates as key molecules for drug resistance, and may be useful as biomarkers of drug-sensitivity.

KLK13 is one member of the tissue kallikrein (KLK) family which includes 15 genes (KLK1-KLK15) and plays a role in tumor cell invasion and migration (13). KLK13 has already been reported to be upregulated in gastric cancer cells after exposure to antineoplastic agents, including epirubicin and methotrexate (14). It has also been reported that overexpression of KLK13 results in an increase of malignant

Table II. List of genes in which expression was changed commonly in drug-resistant cells and biopsy specimens.

Increased in 5FUr and two biopsy specimens after chemotherapy	Decreased in 5FUr and two biopsy specimens after chemotherapy	Increased in CDDPr and two biopsy specimens after chemotherapy	Decreased in CDDPr and two biopsy specimens after chemotherapy	Increased in 5FUr, CDDPr, and two biopsy specimens after chemotherapy	Decreased in 5FUr, CDDPr, and two biopsy specimens after chemotherapy
APOC1	ALPK1	ACTG2	ACOXL	APOC1	ALPK1
BAIAP3	C17orf110	ANPEP	ALPK1	CRYM	CCL21
C4BPA	C4orf47	APOC1	BATF	DNAJC28	CYP2E1
C6orf154	CCL21	C9orf123	BEST4	HSD17B6	ETV7
CAPS2	CYP2E1	CELA3B	CCL21	IQCD	FBXO15
CFTR	ETV7	CRYM	CYP2E1	KLK13	GPR110
CRYM	FBXO15	CTSG	ETV7	KREMEN2	NLRC5
DNAJC28	GPR110	DNAJC28	FBXO15	OLFML3	PLIN4
FRZB	HEPACAM2	HOXB3	GPR110	OTUD7A	SLC22A20
HSD17B6	IFI44L	HSD17B6	INSC	PHACTR3	SLC26A9
IGF1	KRT6C	IQCD	KRT31	PLAT	SLC28A3
IP6K3	LAMC2	KLK13	MUC1	RARRES2	SNORA22
IQCD	NLRC5	KREMEN2	NCKAP5	SRI	
KCTD7	OR52K2	NLRP2	NLRC5	TAC3	
KLK13	PLIN4	NRG1	PCSK9	TNNI3	
KREMEN2	SLC22A20	OLFML3	PLIN4		
LAMA1	SLC26A9	OTUD7A	RAB27B		
LRRC6	SLC28A3	PHACTR3	SLC22A20		
MSLN	SNORA22	PLAT	SLC26A9		
NR2F1	SPRR3	RARRES2	SLC28A3		
OLFML3	ZNF750	RASL10A	SLFNL1		
OOEP		SRI	SNORA22		
OTUD7A		TAC3	SYT13		
PHACTR3		TNFSF9			
PLAT		TNNI3			
PRODH					
RARRES2					
SCN2A					
SEPP1					
SRI					
TAC3					
TNNI3					

5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

cell behavior, and that knockdown of its endogenous gene expression causes a significant decrease in cell migratory and invasive properties (13). We found that KLK13 increased in both drug-resistant cells and biopsy specimens, a finding suggesting that KLK13 may play a role in 5-FU and CDDP resistance in gastric cancer.

In contrast to KLK13, expression of ETV7 decreased in drug-resistant cells. ETV7 is a member of the Ets transcription factor family and is reported to act as an inhibitor of differentiation (15). Since ETV7 was downregulated in drug-resistant gastric cancer in the present study, it may be related with mechanisms of gastric cancer drug-sensitivity.

It has been reported that epigenetic profiles are useful for identifying molecular mediators for cancer drug sensitivity (6). In terms of a correlation between gene expression and DNA methylation, we also performed expression and methylation microarray analyses, and found some genes with altered methylation levels and expression levels. FSCN1 revealed increased expression and hypomethylation in CDDP-resistant cells. FSCN1 has been reported to play an important role in cancer development and is associated with invasion and metastasis (16). It has also been reported that higher intensity FSCN1 staining correlated with more-advanced cancer stages, and inversely correlated with survival rates in

Table III. List of genes in which expression increased and methylation levels decreased, or expression decreased and methylation levels increased.

Increased expression and hypo-methylation in 5FUr	Decreased expression and hyper-methylation in 5FUr	Increased expression and hypo-methylation in CDDPr	Decreased expression and hyper-methylation in CDDPr
ARC	ATP2C2	ABCG4	ATP2C2
C12orf34	C15orf60	C12orf34	C15orf60
CPT1C	PRAME	CARD9	FRMD6
CST6	ZNF773	CST6	SECTM1
CYB5R2		FES	TNFSF12
KCNH8		FSCN1	ZNF773
MESP1		KCNH8	
NOTCH3		MESP1	
RASGRP2		VGF	
TNFSF12			

5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

gastric adenocarcinoma (17). This suggests that FSCN1 may influence patient survival through acquisition of resistance to chemotherapy drugs.

In our experiment, CPT1C was increased in expression in 5-FU and CDDP-resistant cells, and demethylated in 5-FU-resistant cells. CPT1C has been reported to promote tumor growth and rapamycin resistance (18). CPT1C expression correlates inversely with mammalian target of rapamycin (mTOR) pathway activation, contributes to rapamycin resistance in murine primary tumors, and is frequently upregulated in human lung tumors (18). To our knowledge, there has been no report of a relationship between CPT1C and 5-FU or CDDP.

Notch is a transmembrane heterodimeric receptor with 4 distinct members (NOTCH1 to NOTCH4) present in humans. NOTCH3 is one of the Notch family members. It has been reported that NOTCH1, another molecule in Notch family members, expression is associated with cell aggressiveness and 5-FU drug resistance in human esophageal squamous cell carcinoma cell lines *in vitro*, and also with poor survival in human esophageal squamous cell carcinomas (19). It has also been reported that expression levels of Notch3 were increased in rat tracheal epithelial cells after treatment with 5-FU (20). NOTCH3 knockdown enhanced the sensitivity of nasopharyngeal carcinoma cells to CDDP treatment (21), and NOTCH3 overexpression correlated with shorter progression-free/overall survival in patients with advanced stage ovarian carcinoma treated with platinum and taxane (22). In our data, NOTCH3 was upregulated in drug-resistant cells. In gastric cancer, NOTCH3 may be related to drug resistance.

However, we could not find a significant difference in methylation levels among biopsy samples. One of the limitations is that biopsy specimens may not represent the characteristics of the whole tumor, since gastric cancer is known to be biologically heterogeneous.

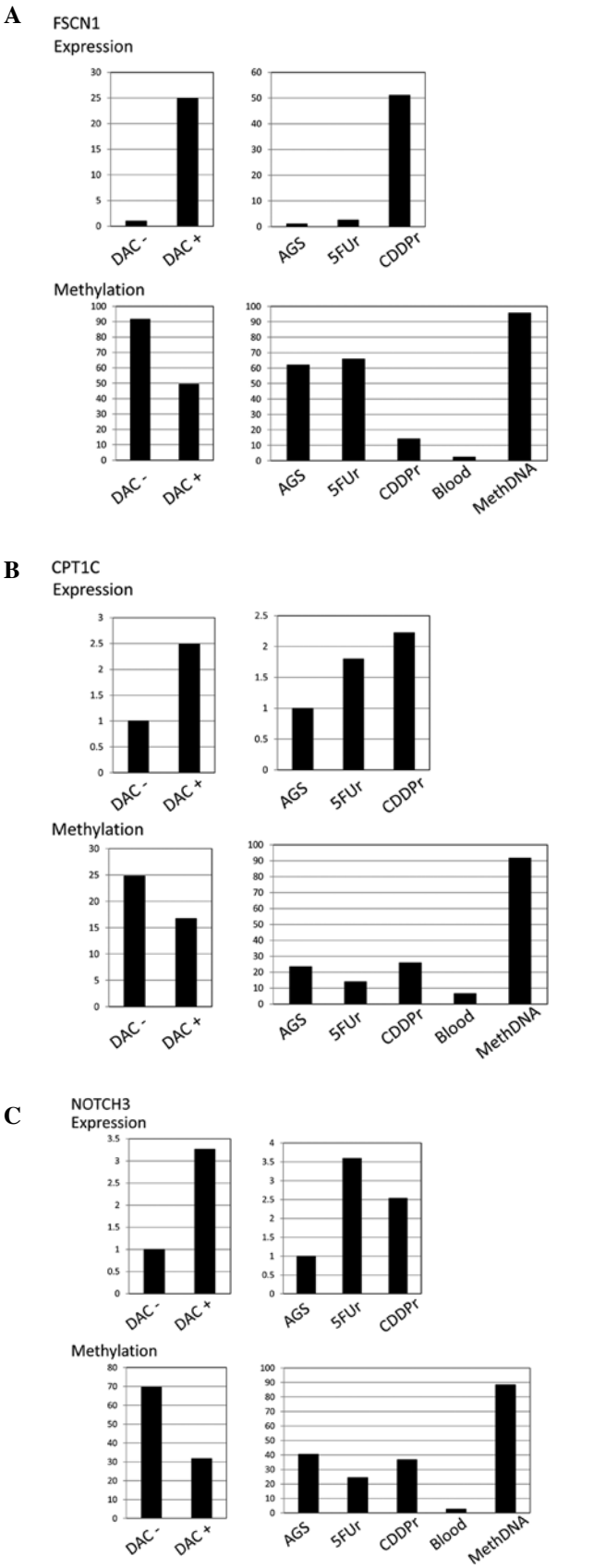

Figure 4. Expression and methylation levels of (A) FSCN1, (B) CPT1C and (C) NOTCH3. Note that AGS cells without treatment with a chemotherapy agent are passaged at the same time as the established drug-resistant cells. Methylation levels may differ between untreated AGS cells using the decitabine treatment experiment and the experiment of comparison with drug-resistant cells. DAC, demethylating agent 5-aza-dC; MethDNA, methylated DNA for positive control; 5FUr, 5-fluorouracil-resistant AGS cells; CDDPr, cisplatin-resistant AGS cells.

In the present study, genes with altered expression and DNA methylation were extracted after treatment with chemotherapeutic agents in gastric cancer. These alterations may be related to mechanisms of gastric cancer drug resistance, and may be useful as biomarkers that predict drug sensitivity. Further studies with a large number of clinical samples are necessary.

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