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Response Predictors of S-1, Cisplatin, and Docetaxel Combination Chemotherapy for Metastatic Gastric Cancer: Microarray Analysis of Whole Human Genes

Shinji Kitamura[a] Toshihito Tanahashi[a] Eriko Aoyagi[a] Tadahiko Nakagawa[a] Koichi Okamoto[a] Tetsuo Kimura[a] Hiroshi Miyamoto[a] Yasuhiro Mitsui[a] Kazuhito Rokutan[b] Naoki Muguruma[a] Tetsuji Takayama[a]

Departments of [a]Gastroenterology and Oncology and [b]Pathophysiology, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan

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Gastric cancer · Microarray · Chemotherapy

Abstract
Objectives: The aim of this study was to identify biomarkers for predicting the efficacy of docetaxel, cisplatin, and S-1 (DCS) therapy for advanced gastric cancer using microarrays of biopsy specimens before chemotherapy. ***Methods:*** Nineteen samples were taken from 19 patients with unresectable metastatic gastric cancer who received DCS as a first-line therapy. Laser capture microdissection was performed, and total cellular RNA was extracted from each microdissected sample. Whole-gene expression was analyzed by microarray, and the difference in mRNA expression observed with the microarrays was confirmed by quantitative real-time PCR. Immunohistochemical staining was performed using clinical tissue sections obtained by endoscopic biopsy. ***Results:*** Eleven patients were identified as early responders and 8 patients as nonresponders to DCS therapy. Twenty-nine genes showed significant differences in relative expression ratios between tumor and normal tissues. A classifier set of 29 genes had high accuracy (94.7%) for distinguishing gene expression between 11 early responders and 8 nonresponders. Decreasing the size of the classifier set to 4 genes (*PDGFB*, *PCGF3*, *CISH*, and *ANXA5*) increased the accuracy to 100%. Expression levels by real-time PCR for validation were well correlated with those 4 genes in microarrays. ***Conclusion:*** The genes identified may serve as efficient biomarkers for personalized cancer-targeted therapy.

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Introduction

Gastric cancer is the leading cause of cancer-related death worldwide and is a major health problem in Southeast Asian countries including Japan [1, 2]. Although mortality from gastric cancer has been declining recently due to improvements in early diagnosis and treatment, the prognosis of patients with advanced gastric cancer (AGC) remains poor.

The efficacy of various combinations of fluoropyrimidine, irinotecan, platinum drugs, and anthracyclines has been investigated for the treatment of metastatic gastric cancers. Webb et al. [3] performed a prospective randomized study that compared the efficacy of a combination of epirubicin, cisplatin, and protracted venous infusion with

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Prof. Tetsuji Takayama
Department of Gastroenterology and Oncology
Institute of Biomedical Sciences, Tokushima University Graduate School
Tokushima 770-8503 (Japan)
E-Mail takayama@tokushima-u.ac.jp

fluorouracil (5-FU) (ECF) with the standard combination of 5-FU, doxorubicin, and methotrexate (FAMTX) in previously untreated patients with advanced esophagogastric cancer. The ECF regimen resulted in a survival and response advantage, tolerable toxicity, and better quality of life and cost-effectiveness compared with FAMTX chemotherapy. Van Cutsem et al. [4] conducted a phase III study of docetaxel and cisplatin plus 5-FU compared with cisplatin and 5-FU as a first-line therapy for AGC. Addition of docetaxel to cisplatin and 5-FU significantly improved the time to progression, survival, and response rate in gastric cancer patients but resulted in some increases in toxicity. Recently, Kilickap et al. [5] suggested that combination therapy with docetaxel/cisplatin/5-FU is superior in terms of overall response rate and progression-free survival as compared to cisplatin/5-FU and ECF combinations in AGC.

We have reported that a triple-drug combination chemotherapy regimen consisting of docetaxel, cisplatin, and S-1 (DCS) is well tolerated in patients with metastatic gastric cancer and has a very high response rate (87.1%), a high downstaging rate (25.8%), and a high curative surgery rate (22.6%) [6, 7]. A subsequent phase II study of this regimen also showed a high response rate (81%) [8]. In a phase II study of neoadjuvant chemotherapy, preoperative treatment with DCS for localized AGC demonstrated a sufficient R0 resection rate and a good pathological response with manageable toxicities [9], and a phase III trial is currently in progress. Although DCS therapy shows very high efficacy for metastatic gastric cancer, there still are unresponsive cases, and grade 3–4 neutropenia and leukocytopenia are often observed. Therefore, it is very important to discover biomarkers to aid in the selection of appropriate candidates for this therapy. In this study, we conducted a clinicopathological analysis to identify biomarkers for predicting the efficacy of DCS using microarray analysis of biopsy samples before chemotherapy.

Materials and Methods

Patients, Samples, and Study Design

Patients with unresectable metastatic gastric cancer (clinical stage III or IV) who received DCS as a first-line therapy were retrospectively screened. The patients were classified into 3 groups based on tumor regression rates (TRR) according to the Response Evaluation Criteria in Solid Tumors (RECIST) [10] after 2 cycles of treatment: an early responder group showing >20% TRR, a responder group showing 10–20% TRR, and a nonresponder group showing <10% TRR [11, 12]. There were no patients who showed >20% TRR after 3 cycles or more. Prior to chemotherapy, all patients underwent biopsy from tumor and background gastric mucosa under endoscopic examination. Tumor samples for histologic examination were fixed in 10% buffered formaldehyde, and tumor samples for gene expression analysis were frozen in liquid nitrogen and stored at –80 °C until use.

Treatment with DCS Combination Therapy

S-1 was administered orally twice daily on days 1–14 at a dose of 60 mg/m^2. Docetaxel 50 mg/m^2 was given by intravenous infusion for 1.5 h. Subsequently, cisplatin was administered by intravenous infusion for 2 h at 60 mg/m^2 in saline solution on day 8. Cycles were repeated every 3 weeks. To avoid cisplatin-induced renal damage, patients were hydrated on days 7–9 with 2,000 mL of 5% dextrose in 0.9% sodium chloride. Prophylactic administration of antiemetic medication (5-HT$_3$ antagonist, corticosteroid, and aprepitant) at a standard dose was routinely used to prevent nausea and vomiting when cisplatin was administered. Granulocyte colony-stimulating factor was administered when grade 4 neutropenia or grade 3 or 4 neutropenia with high-grade fever were observed.

Microdissection

Laser capture microdissection was performed on 19 frozen tissue samples including gastric cancer lesions and normal tissue in the background mucosa. We stained thick frozen sections with hematoxylin and eosin and selectively collected cancer cells and normal cells under an inverted microscope (CKX41, Olympus, Tokyo, Japan) incorporated in a laser microdissection system (MMI CellCut, Molecular Machines and Industries Germany), which was designed for quick and precise isolation of cells and tissue.

RNA Extraction and Microarray Analysis

Total cellular RNA was extracted from each microdissected sample. The quality of the purified RNA was assessed with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, CA, USA). Total RNA (50 ng) was amplified with WT-Ovation FFPE RNA Amplification System V2 (NuGEN, San Carlos, CA, USA) and biotin labeled with FL-Ovation cDNA Biotin Module V2 (NuGEN). Prepared cDNA was added to a whole human genome oligo DNA microarray (4 × 44 k; Agilent). Hybridization was performed at 65 °C for 17 h. After washing, fluorescence intensity was assayed using a scanner (G2565BA; Agilent). The signal intensities of Cy3 were quantified and analyzed by subtracting background using Feature Extraction (Agilent). GeneSpring GX10.0.2 analysis software (Agilent) was used to normalize data and to identify 13,174 genes with fluorescence intensities >100 in at least 1 RNA sample. The complete datasets were deposited in the Gene Expression Omnibus database (accession No. GSE31811).

Microarray Data Analysis

Microarray data were analyzed using the GX10.0.2 software. In all experimental groups, differentially expressed genes were identified by a significance level of $p < 0.01$ and a threshold level of a >3-fold change. The prediction scores of all samples were obtained by a leave-one-out approach, in which one sample at a time was removed from the sample set [13]. We also used N-fold cross-validation [14]. The classes in the input data are randomly divided into N equal parts; $N - 1$ parts are used for training, and the remaining 1 part is used for testing.

Quantitative Real-Time PCR of mRNA

The difference in mRNA expression observed with the microarrays was confirmed by quantitative real-time (qRT)-PCR analysis of individual samples. Equal amounts of DNA-free RNA from each sample were used. qRT-PCR was performed using an ABI 7500 system with specific primer and probe sets for the TaqMan probe detection assay (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. β-Actin was used as an endogenous quantity control.

Immunohistochemical Analysis

To confirm the differential expression of marker genes between early responders and nonresponders, clinical tissue sections obtained by endoscopic biopsy were stained using LSAB™ 2 Kit/HRP (DAKO, Tokyo, Japan). Briefly, after endogenous peroxidase and protein blocking reactions, the primary antibodies of PDGFB (ab23914, Abcam, Cambridge, UK), PCGF3 (HPA018487, Sigma-Aldrich, St. Louis, MO, USA), cytokine-inducible SH2-containing protein (CISH; sc-74581, Santa Cruz Biotechnology Inc., Dallas, TX, USA), or annexin A5 (ANXA5; H00000308-MO1, Abnoba, Taiwan) were added, followed by HRP-labeled anti-rabbit or anti-mouse IgG as the secondary antibody. The specimens were counterstained with hematoxylin. Positivity of immunostaining was assessed as absent or present by 2 independent investigators without prior knowledge of the clinical follow-up data. Cases were accepted as positive only when reviewers independently defined them as such.

Statement of Ethics

This study was approved by the Institutional Review Board of Tokushima University Hospital. Written informed consent was obtained from each patient before they underwent tumor biopsy.

Results

Characteristics of the Patients

A total of 19 patients were enrolled in this study. According to RECIST criteria [10], 11 patients were identified as early responders and 8 patients as nonresponders by 3 board-certified gastroenterologists. There were no patients in the responder group showing 10–20% TRR. Characteristics of the 11 early responders and 8 nonresponders are shown in Table 1. The mean age of the 19 cases (11 male, 8 female) was 59.9 years (range 33–76). There was no statistical difference between the 2 groups. Histopathological types were poorly differentiated adenocarcinoma in 17 cases and differentiated adenocarcinoma in 2 cases.

Identification of Genes Associated with Sensitivity to DCS Treatment

To extract differentially expressed genes of 11 early responders and 8 nonresponders, we compared the expression levels of 13,174 genes after quality checking. The relative expression ratios between tumor and normal tissues in each patient were calculated. We found 29 genes that showed significant differences in relative expression ratios between tumor and normal tissues (unpaired t test, $p < 0.01$), and these genes showed 3-fold or greater changes. The 29 genes are listed in Table 2.

Table 1. Patient characteristics

	Early Responder	Non-responder
Total patients	11	8
Median age (range), years	59.5 (34–76)	60.6 (33–75)
Sex		
Male	4	7
Female	7	1
Performance status		
0	7	5
1	4	3
Differentiation		
Intestinal	2	0
Diffuse	9	8
Clinical stage		
III	0	2
IV	11	6

Prediction of the Efficacy of DCS Treatment

On the basis of the expression profiles of the 29 genes, we analyzed their potential for predicting the efficacy of DCS treatment. Hierarchical clustering of the 29 genes comparing the early responder group and the nonresponder group is shown in a heat map (Fig. 1). The green or red colors of each block represent the normalized gene expression levels. Prediction scores were calculated according to the procedures described in the Materials and Methods section. A classifier set of 29 genes showed high accuracy (94.7%) for distinguishing gene expression between the 11 early responders and the 8 nonresponders, as evaluated by the leave-one-out algorithm. With the N-fold approach, a similarly high classification accuracy (94.7%) was obtained with the 29 gene sets (Fig. 2; Table 3). Decreasing the size of the classifier set to 4 genes (*PDGFB*, *PCGF3*, *CISH*, and *ANXA5*) increased the accuracy to 100% with both the leave-one-out and N-fold methods (Table 4). *PDGFB*, *PCGF3*, *CISH*, and *ANXA5* genes were differentially expressed in early responders and nonresponders and had a good predictive accuracy for the classification of treatment outcome.

Table 2. List of 29 candidate genes for discriminating early responders from nonresponders to DCS therapy

ID	Symbol	Gene name	*p* value	Regulation
1	PCGF3	Homo sapiens polycomb group ring finger 3 (PCGF3)	<0.01	down
2	STYX	Serine/threonine/tyrosine-interacting	<0.01	down
3	Q9H7N0 HUMAN	FAM39B protein	<0.01	up
4	B4GALT5	Homo sapiens UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	<0.01	down
5	EGR1	Homo sapiens early growth response 1	<0.01	down
6	BC062753	Homo sapiens cDNA clone IMAGE: 3933366, partial cds	<0.01	up
7	PSMD11	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	<0.01	up
8	PDGFB	Homo sapiens platelet-derived growth factor beta polypeptide	<0.01	up
9	HECA	Homo sapiens headcase homolog	<0.01	down
10	DB365607	DB365607 NT2RP2 Homo sapiens cDNA clone NT2RP2003447 3′, mRNA sequence	<0.01	down
11	ANTXR2	Homo sapiens anthrax toxin receptor 2	<0.01	down
12	ATP7B	Homo sapiens ATPase, Cu++ transporting, beta polypeptide	<0.01	down
13	ANXA5	Homo sapiens annexin A5	<0.01	down
14	ENST00000373575	Eukaryotic translation initiation factor 4E type 2	<0.01	up
15	CISH	Homo sapiens cytokine inducible SH2-containing protein	<0.01	down
16	PLK2	Homo sapiens polo-like kinase 2	<0.01	down
17	E01979	xq40c08.x1 NCI_CGAP_Lu28 Homo sapiens cDNA clone IMAGE:2753102 3′ similar to gb:X57352 INTERFERON-INDUCIBLE PROTEIN 1-8U (HUMAN);, mRNA sequence [AW275876]	<0.01	down
18	TRBV5-4	Homo sapiens T cell receptor beta variable 5-4, mRNA	<0.01	up
19	ENST00000307437	PREDICTED: Homo sapiens similar to ribosomal protein S12	<0.01	down
20	ERLIN1	Homo sapiens ER lipid raft associated 1	<0.01	down
21	LOC441073	PREDICTED: Homo sapiens similar to 60S ribosomal protein L26	<0.01	down
22	JMJD2A	Homo sapiens jumonji domain containing 2A	<0.01	up
23	HP1BP3	Homo sapiens heterochromatin protein 1, binding protein 3	<0.01	up
24	THC2535233	Q4T2V2_TETNG (Q4T2V2) Chromosome 10 SCAF10171, whole genome shotgun sequence	<0.01	down
25	AVPI1	Homo sapiens arginine vasopressin-induced 1	<0.01	down
26	DB329052	DB329052 PLACE7 Homo sapiens cDNA clone PLACE7013977 3′, mRNA sequence	<0.01	down
27	MYCBP2	Homo sapiens MYC binding protein 2	<0.01	up
28	FAM116A	Homo sapiens family with sequence similarity 116, member A	<0.01	down
29	AP1S1	Homo sapiens adaptor-related protein complex 1, sigma 1 subunit	<0.01	down

DCS, docetaxel, cisplatin, and S-1.

Validation of mRNA Levels of PDGFB, PCGF3, CISH, and ANXA5

To validate the expression levels of PDGFB, PCGF3, CISH, and ANXA5 mRNA in the early responder and nonresponder groups, we performed TaqMan qRT-PCR and compared expression levels with signal intensity by microarray (Fig. 3). We obtained expression ratios between tumor and normal tissues. In the analysis of all 19 subjects, we found high, moderate, and weak correlations with CISH (r^2 = 0.567), PDGFB (r^2 = 0.338), and PCGF3 (r^2 = 0.248) but not with ANXA5 (r^2 = 0.162). The data obtained by TaqMan qRT-PCR were well correlated with those from microarray analysis.

Immunohistochemical Validation of PDGFB, PCGF3, CISH, and ANXA5

To validate the differential expression of predictive protein markers in the early responder and nonresponder groups, we carried out immunohistochemical staining with antibodies for PDGFB, PCGF3, CISH, and ANXA5 (Fig. 4). In the case of nonresponders, expression of PDGFB was low, but CISH, ANXA5, and PCGF3 were highly expressed. In contrast, expression of PDGFB was increased, but resistant markers of CISH, ANXA5, and PCGF3 were decreased in early responders. These immunohistochemical results were consistent with the microarray data.

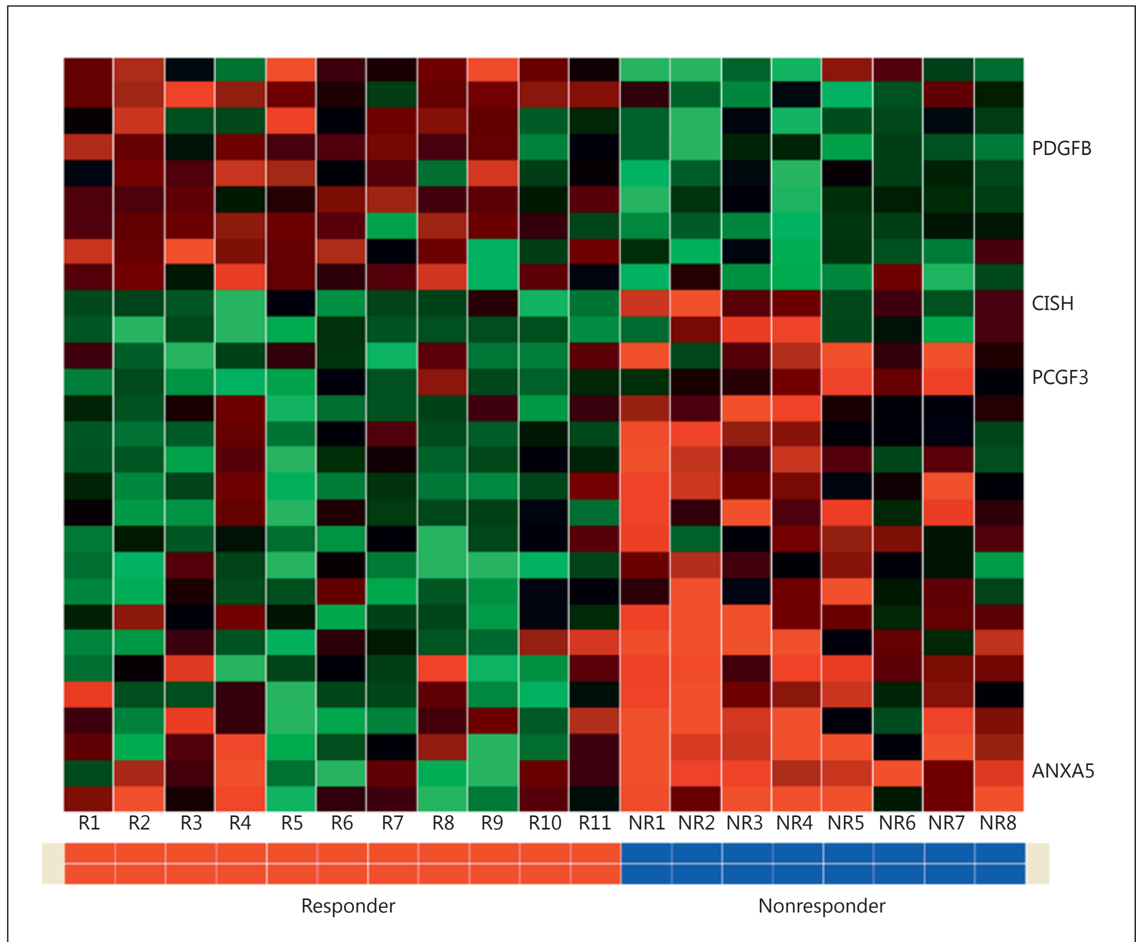

Fig. 1. Hierarchical clustering of the 29 genes comparing the early responder and nonresponder groups is shown in a heat map. High expression is shown in red and low expression in green. Early responders and nonresponders are well distinguished. R, responders; NR, nonresponders.

Table 3. Validation in 29 genes

	Prediction		
	effective	noneffective	accuracy
Leave-one-out validation			
True effective	11	0	100%
True noneffective	1	7	87.50%
Total (18/19)			94.70%
N-fold validation			
True effective	11	0	100%
True noneffective	1	7	87.50%
Total (18/19)			94.70%

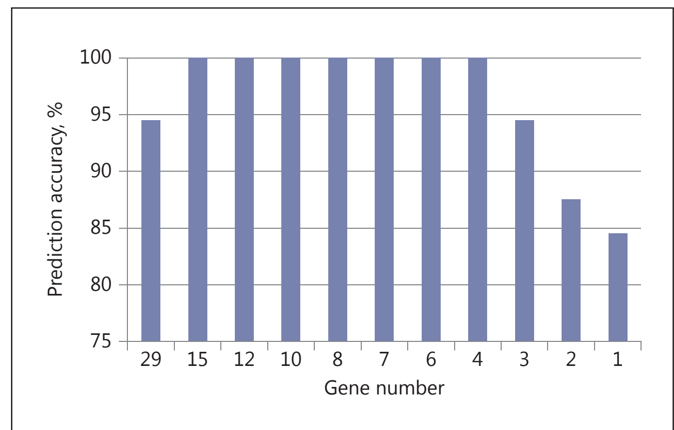

Fig. 2. Prediction accuracy by gene number. The prediction accuracy was 100% with only 4 genes.

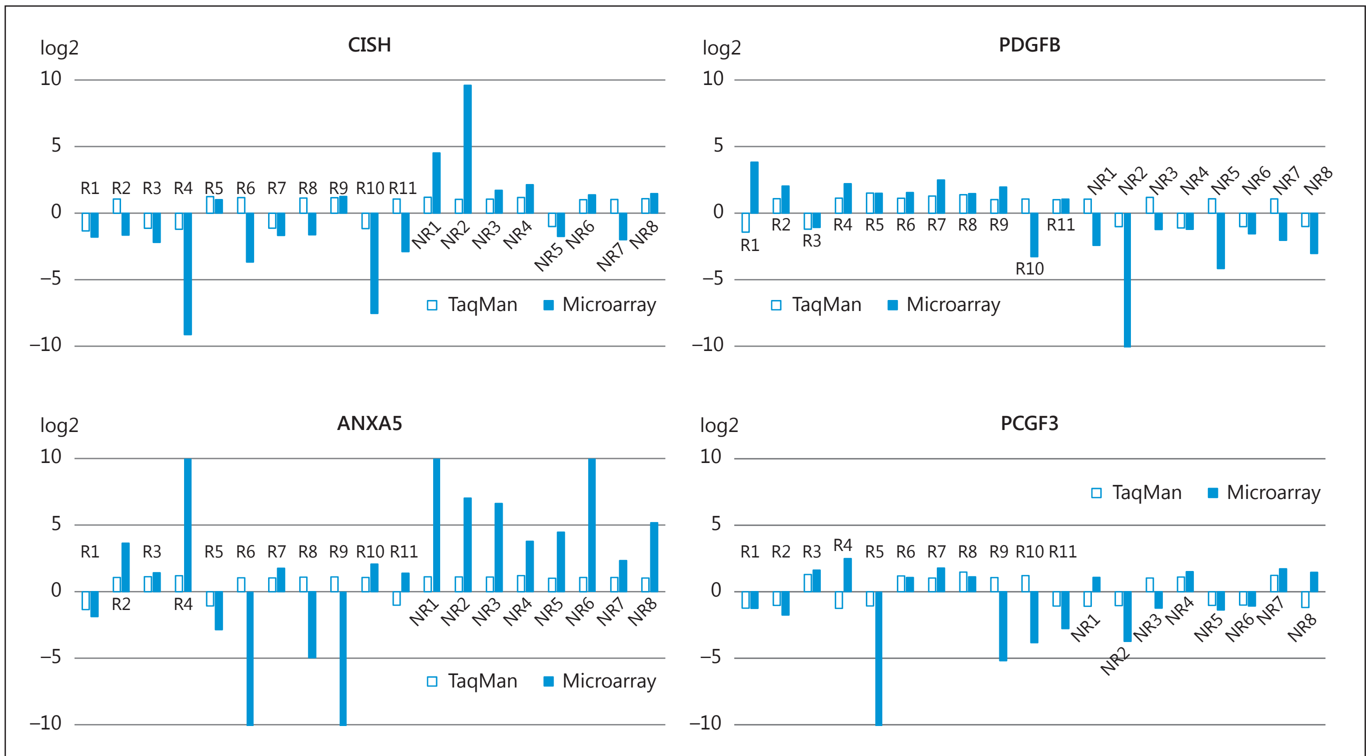

Fig. 3. Comparison of quantitative real-time PCR and microarray expression of PDGFB, PCGF3, CISH, and ANXA5. Expression ratios between tumor and normal tissues are shown. The results are indicated as white (TaqMan) and blue bars (microarray). R, responders; NR, nonresponders.

Discussion

Recent studies using microarray analysis have demonstrated a relationship between gastric cancer progression, metastasis, and genetic expression; however, there are few reports regarding sensitivity or resistance to anticancer drugs [15, 16]. In this study, we conducted a clinicopathological investigation to identify biomarkers for predicting the efficacy of DCS therapy, which is the most effective regimen for AGC, using biopsy samples taken before chemotherapy and microarray analysis. We determined the whole genome sequence and investigated the genome-affecting sensitivity and resistance to DCS therapy.

Gastric cancer tissues are composed of complex admixtures of different cell types including inflammatory and necrotic cells, and the procurement of pure samples is indispensable for identifying causative genes. Thus, laser capture microdissection was performed, and total cellular RNA was extracted from microdissected samples. As a result, 29 genes affecting the therapeutic efficacy of DCS were identified. The classifier set of 29 genes showed high accuracy (94.7%) for distinguishing between the 11 early responders and the 8 nonresponders using both the leave-one-out and *N*-fold algorithms. Decreasing the size of the classifier set to 4 genes (*PDGFB*, *PCGF3*, *CISH*, and *ANXA5*) increased the accuracy to 100% with both methods. Expression patterns of these 4 genes in the early responder and nonresponder groups were correlated with

Table 4. Validation in 4 genes

	Prediction		
	effective	noneffective	accuracy
Leave-one-out validation			
True effective	11	0	100%
True noneffective	0	8	100%
Total (19/19)			100%
N-fold validation			
True effective	11	0	100%
True noneffective	0	8	100%
Total (19/19)			100%

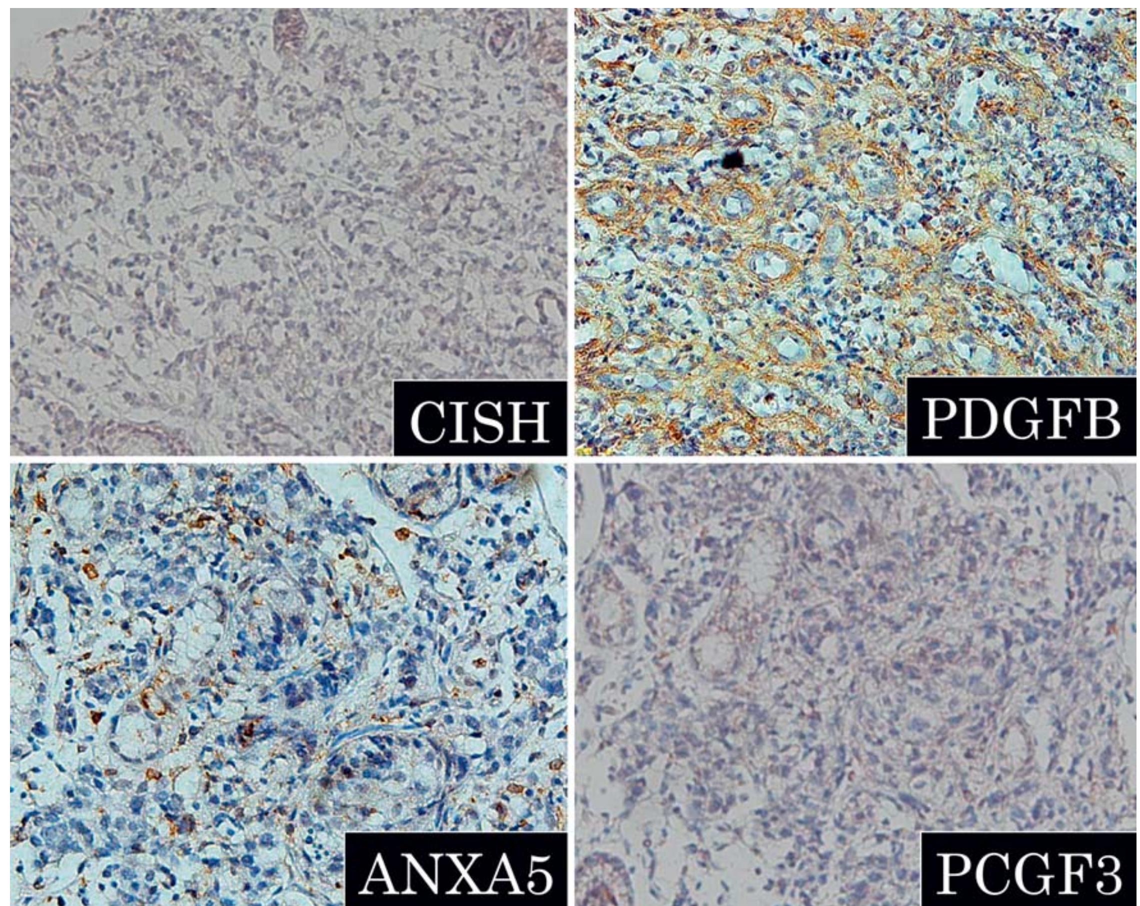

Fig. 4. Immunohistochemical staining for PDGFB, PCGF3, CISH, and ANXA5 in a specimen from an early responder case. Expression of PDGFB was high, but expression of CISH, ANXA5, and PCGF3 was low.

the treatment outcome. The results demonstrate a potentially predictive value in DCS therapy.

PDGFB is a member of the platelet-derived growth factor family and is known as an important factor in neoplasms, such as lung, breast, and colorectal cancers [17]. PDGF contributes to cancer development and progression by both autocrine and paracrine signaling mechanisms. It is also well known that cancer development is often associated with neovascularization [18], and PDGF also plays a role in vascular development. Our results showed that PDGF was a sensitive marker of DCS efficacy, and these characteristics are presumably the reason why PDGF is predictive of DCS efficacy.

CISH protein is encoded by the *CISH* gene and is a known suppressor of cytokine signaling (SOCS), i.e., a STAT-induced STAT inhibitor [19]. With respect to cancer, CISH protein has been hypothesized to act as an activator of the MAPK pathway and as an inhibitor of differentiated cell functions mediated through the JAK/STAT pathway. The role of CISH protein in tumor progression (i.e., positive effects on cell proliferation and colony formation) has been demonstrated [20].

PCGF3, the protein encoded by this gene, contains a C3HC4-type RING finger, which is a motif known to be involved in protein-protein interactions. The specific function of this protein has not yet been determined and is poorly described in the field of oncology. It has recently been reported that RING1 protein expression may be a favorable independent prognostic parameter for non-small cell lung cancer [21], but it is unclear how this molecule may be involved in tumor proliferation and resistance to anticancer drugs in patients with gastric cancer.

Annexins are important in various cellular and physiological processes and have been shown to be involved in trafficking and organization of vesicles, exocytosis, endo-

cytosis, and calcium ion channel formation [22]. One type of annexin, ANXA2, is involved in gastric cancer multidrug resistance via regulation of the p38MAPK and AKT pathways, as well as in multidrug resistance [23]. ANXA5 is a potentially predictive marker in tumors, and it promotes a variety of cancers. It is involved in metastasis, invasion, angiogenesis, and cancer progression. ANXA5 deregulation is associated with drug resistance in nasopharyngeal carcinoma and gastric cancer, and the cell sensitivity to cisplatin, paclitaxel, and 5-FU is increased by 36-, 17-, and 4-fold, respectively, following the downregulation of ANXA5 [24].

We focused on the aforementioned combination of 4 proteins and conducted immunohistochemical staining for the samples after DCS therapy. We had effective cases showing positive PDGFB and negative CISH, PCGF3, and ANXA5 responses (Fig. 4) as well as noneffective cases showing negative PDGFB and positive CISH, PCGF3, and ANXA5 responses. This confirms that the efficacy of DCS therapy can be predicted using immunohistochemistry of biopsy samples. These data suggest that the patients judged as early responders should receive DCS therapy as the first-line treatment. In contrast, nonresponders should receive other forms of therapy, such as irinotecan-based or paclitaxel-based regimens. Thus, our results may contribute to the development of personalized medicine for unresectable metastatic gastric cancer.

There may, however, be some heterogeneity in the results of microarray, qPCR, and immunohistochemical staining. This heterogeneity was induced by the difference between the superficial and the deep portion of the tumor, and the result of the microarray may not demonstrate the true properties of the tumor. Considering the molecular heterogeneity of gastric cancer, further analysis of expression patterns and investigation using a scoring system for expression grade is required to validate our results. It is also necessary to elucidate how these molecules affect the anticancer drugs docetaxel, cisplatin, and S-1 independently. Moreover, the relationship between the expression patterns of the 4 genes should be evaluated in a large-scale study of gastric cancer patients.

In conclusion, we identified a group of genes in human tissue samples that are predictive of the therapeutic efficacy of anticancer drugs for the treatment of unresectable AGC. These genes may serve as efficient biomarkers for personalized cancer-targeted therapy. Further investigations are needed to evaluate response rates, adverse effects, and survival period.

Disclosure Statement

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