Predicting response of bladder cancers to gemcitabine and carboplatin neoadjuvant chemotherapy through genome-wide gene expression profiling

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Abstract. Neoadjuvant chemotherapy with gemcitabine and carboplatin (GC) for invasive bladder cancer increases the chance of a radical response for a subset of patients, while other patients suffer from severe adverse drug reactions without any benefit. To establish a method for predicting the response to chemotherapy with GC, the expression profiles of biopsy samples from 37 advanced bladder cancers were analyzed using a microarray consisting of 38,500 genes or ESTs. Upon analysis of 9 'responder' and 9 'non-responder' tumors, 12 'predictive' genes were found to be significantly differentially expressed between the 'responder' and 'non-responder' groups, and a numerical prediction scoring system that clearly separated the responder group from the non-responder group was established. This system accurately predicted the drug responses of 18 of 19 additional test cases that were reserved from the original 37 cases. Moreover, a quantitative PCR-based prediction system was developed that may be feasible for routine clinical use, and the sensitivity of invasive bladder cancer to neoadjuvant chemotherapy with GC was able to be predicted by the expression patterns in this set of genes. Nearly 50% of patients treated with GC or methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC) therapy have been reported to achieve complete or partial response to either of these therapies. When we applied this prediction system as well as the system for M-VAC, we expected that approximately 80% of the patients would achieve significant tumor shrinking (>60%) by selection of either the GC or M-VAC regimens. Our results suggest that the two prediction scoring systems lead to achievement of 'personalized therapy' for the treatment of invasive bladder cancer and should improve the quality of life for patients with this disease.

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Abbreviations: GC, gemcitabine and carboplatin; M-VAC, methotrexate, vinblastine, doxorubicin and cisplatin; PR, partial response; PS, performance status; AUC, area under the curve; RT-PCR, reverse transcription-polymerase chain reaction

Key words: gemcitabine, carboplatin, bladder cancer, neoadjuvant chemotherapy, prediction, gene expression profile, microarray

Introduction

Bladder cancer is the second most common genitourinary tumor, having an incidence of 357,000 new cases each year worldwide; approximately one-third of these cases are likely to be invasive or metastatic disease at the time of diagnosis (1). Although radical cystectomy is considered as the gold standard for the treatment of patients with localized, but muscle-invasive, bladder cancer, nearly 50% of patients develop metastases within 2 years after cystectomy and subsequently die of the disease (2).

Neoadjuvant chemotherapy is usually applied to muscle-invasive bladder cancer to manage micrometastases and improve prognosis (3,4). A neoadjuvant chemotherapy regimen involving gemcitabine and carboplatin (GC) as well as that of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC), followed by radical cystectomy, have been found to decrease the recurrence rate compared to radical cystectomy alone and to improve survival (5-8). Furthermore, a small subset of patients who respond well to neoadjuvant chemotherapy may have a chance to preserve bladder function and maintain a good quality of life.

However, since no method yet exists for predicting the response of an individual patient to GC therapy, some patients suffer from adverse reactions to these drugs without achieving any benefit in terms of a positive effect. They often are not able to undergo additional treatment as their physical condition deteriorates (9,10). Hence, the development of a reliable method to predict the effectiveness of a specific therapy is critical to assign the appropriate treatment to individual patients with bladder cancer. Various factors have

been reported to be associated with chemosensitivity or prognosis, yet each of these factors is not sufficiently effective to reliably predict individual response. Since cancer cells in individual patients are characterized by a large number of features, a larger body of information is required to precisely characterize them. The profiling of gene expression patterns on genome-wide microarrays enables investigators to perform comprehensive analyses of complex molecular activities in cancer cells. Systematic analysis of the expression levels of thousands of genes is also a useful approach for identifying molecules related to response to chemotherapy or radiation therapy.

In this study, we report the establishment of a system for predicting response to GC neoadjuvant chemotherapy for patients with invasive bladder cancer using genome-wide information obtained for 37 cases using a microarray consisting of 38,500 genes. This was carried out in combination with laser microbeam microdissection of the tumors to enrich the proportion of cancer cells for accurate analysis. We identified 12 genes that exhibited significantly different levels of expression between responders and non-responders who received a neoadjuvant GC regimen. In addition, although the response rate to each of the M-VAC and GC regimens was reported to be nearly 50%, we suggest that the personalized selection of an appropriate chemotherapy regime using prediction systems for the response to these regimens may improve the response rate to approximately 80%. Our results strongly imply that 'personalized therapy' based on expression levels of a small number of genes may improve the quality of life of a larger proportion of patients with invasive bladder cancer.

Materials and methods

Patients, tissue samples and neoadjuvant chemotherapy. Bladder cancer tissue samples from punch biopsy and corresponding clinical information were obtained from the Iwate Medical University after each patient provided written informed consent. A total of 37 cancer samples from patients (6 females and 31 males; median age, 67 years; age range, 52-78 years) (Table I) histologically confirmed as having transitional cell carcinoma of the bladder were selected for this study. The clinical stage of each patient was judged according to the International Union Against Cancer Tumor-Node-Metastasis classification; we enrolled only patients who had no node metastasis at clinical stages T2aN0M0 to T4aN0M0 and were expected to undergo radical cystectomy without prior radiation therapy. All participants had no serious abnormality in renal, hepatic or hematologic function and an Eastern Cooperative Oncology Group performance status (PS) judged to be ≤2. Three to five pieces of cancer tissue were obtained from each patient at the time of biopsy before neoadjuvant chemotherapy. These samples were immediately embedded in TissueTek OCT compound (Sakura, Tokyo, Japan), frozen and stored at -80˚C. The frozen tissues were sliced into 8-μm sections using a cryostat (Sakura) and were then stained with H&E for histologic examination. Bladder cancer cells were selectively enriched for our experiments using the EZ-cut system with a pulsed UV narrow beam focus laser (SL Microtest GmbH, Germany) according to the manufacturer's protocols. All patients were examined through chest X-ray, computed tomography and magnetic resonance imaging of their abdomen and pelvis and were confirmed to have neither lymph node nor distant metastases. Patients were administered two 28-day cycles of GC neoadjuvant chemotherapy as follows: gemcitabine (1,000 mg/m^2) on days 1, 8 and 15; carboplatin (5 AUC) on day 2. According to their responses to the treatment, we categorized the patients into two groups: 'responders' who achieved significant tumor shrinking (>60%) after two courses of GC neoadjuvant chemotherapy, and 'non-responders' who revealed no significant tumor shrinking (≤60%) after the two courses of chemotherapy.

GeneChip hybridization. The Affymetrix human genome U133 Plus 2.0 GeneChip arrays were used for microarray hybridizations. This GeneChip comprises >54,000 probe sets and analyzes the expression level of 47,400 transcripts. For microarray hybridization, we followed the protocol described in the Affymetrix GeneChip 3'IVT Express Kit User Manual Protocol (Affymetrix, Santa Clara, CA, USA). For the synthesis of single-strand cDNA, 30-150 ng of total RNA was reversely transcribed using the First-Strand Enzyme mix and Buffer mix included in the GeneChip 3'IVT Express kit and double-strand cDNA synthesis according to the manufacturer's instructions followed by the Second-Strand Enzyme mix and Buffer mix included in the GeneChip 3'IVT Express kit. IVT amplification generates multiple copies of biotin-modified aRNA from the double-stranded cDNA templates (Affymetrix). A 15-μg aliquot of the labeled product was fragmented by heat and ion-mediated hydrolysis at 94˚C for 35 min in H_2O and 8 μl of 5X fragmentation buffer (Affymetrix). The fragmented cRNA was hybridized for 16 h at 45˚C in a Hybridization Oven 640 to a U133 Plus 2.0 oligonucleotide array (Affymetrix). The washing and staining of the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR, USA) was completed in a Fluidics Station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip scanner 3000 (Affymetrix).

Data analysis. The obtained image files were analyzed with the Affymetrix data suite system, Microarray Suite 5.0 (MAS 5.0; Affymetrix). Global normalization at a target value of 500 was applied to all 38 arrays (37 cancer arrays and one array of the universal control) using the Affymetrix® GeneChip® Command Console 1.1 (Affymetrix). Normalized data from text files were imported to a Microsoft Excel spread sheet. Since data derived from low-signal intensities are less reliable, we excluded transcripts with low intensities from further analysis when the signal intensities of both the normal and cancer cells were lower than that of the cut-off. For the other genes, we calculated the signal intensities of the cancer/normal ratio using the raw data of each sample (11).

Hierarchical clustering analysis. We used web-available software ('Cluster' and 'TreeView') written by M. Eisen (http://genome-www5.stanford.edu/MicroArray/SMD/restech.html) to create a graphic representation of the microarray data and to create a dendrogram of hierarchical clustering. Before the clustering algorithm was applied, the fluorescence ratio for each spot was first log-transformed, and then the data for each sample were median-centered to remove experimental biases.

Table I. Clinicopathological features of the examined bladder cancer patients.

ID no.	Gender	Age	Stage	Grade	Response	Prediction	Post treatment
BCGC1006	M	60	T4	G3	Responder	Learning	Cystectomy
BCGC1007	F	71	T3a	G2	Responder	Learning	Cystectomy
BCGC1010	M	67	T3b	G3	Responder	Learning	Cystectomy
BCGC1011	M	69	T3b	-	Responder	Learning	Cystectomy
BCGC1016	M	53	T3a	G2	Responder	Learning	Cystectomy
BCGC1020	F	62	T2a	G3	Responder	Learning	Cystectomy
BCGC1021	M	67	T3a	G2	Responder	Learning	Cystectomy
BCGC1022	M	71	T2a	G3	Responder	Learning	Cystectomy
BCGC1029	M	56	T3b	G3	Responder	Learning	Cystectomy
BCGC1005	M	56	T2b	G1	Non-responder	Learning	Cystectomy
BCGC1009	M	60	T4	G3	Non-responder	Learning	Radiation
BCGC1015	M	78	T3a	G2	Non-responder	Learning	Cystectomy
BCGC1017	M	59	T3a	G3	Non-responder	Learning	Cystectomy
BCGC1018	M	72	T3a	G2	Non-responder	Learning	Cystectomy
BCGC1024	M	67	T2a	G2	Non-responder	Learning	Cystectomy
BCGC1025	M	52	T2b	G3	Non-responder	Learning	Radiation
BCGC1026	M	62	T4	G3	Non-responder	Learning	Radiation
BCGC1027	M	62	T3b	G3	Non-responder	Learning	Radiation
BCGC1001	M	60	T4	G1	Responder	Test	Cystectomy
BCGC1003	M	64	T3b	G3	Responder	Test	Cystectomy
BCGC1012	M	67	T2a	G3	Responder	Test	Cystectomy
BCGC1013	M	74	T3b	G2	Responder	Test	Cystectomy
BCGC1014	M	76	T3b	G2	Responder	Test	Cystectomy
BCGC1019	F	71	T2a	G2	Responder	Test	TUR-Bt
BCGC1031	M	75	T2a	G3	Responder	Test	Cystectomy
BCGC1033	F	71	T2a	G2	Responder	Test	Cystectomy
BCGC1036	M	71	T3b	G2	Responder	Test	Cystectomy
BCGC1041	M	68	T2b	G3	Responder	Test	Cystectomy
BCGC1045	M	67	T2b	G3	Responder	Test	GCx1
BCGC1030	M	55	T2b	G2	Non-responder	Test	Cystectomy
BCGC1032	M	70	T2a	G3	Non-responder	Test	Radiation
BCGC1034	F	59	T2a	G2	Non-responder	Test	-
BCGC1037	M	66	T2a	G2	Non-responder	Test	-
BCGC1039	M	73	T3a	G2	Non-responder	Test	-
BCGC1047	M	71	T4	G3	Non-responder	Test	Radiation
BCGC1048	M	74	T3b	G2	Non-responder	Test	GCx1
BCGC1049	F	70	T4	G3	Non-responder	Test	GCx1

Response, response to neoadjuvant GC treatment. Responder, patient who achieved significant tumor shrinking (>60%) after two courses of GC neoadjuvant chemotherapy; Non-responder, patient who did not achieve significant shrinking of the tumors (≤60%) after two courses of chemotherapy. Learning, samples used to develop the prediction system. Test, samples used for test cases. TUR-Bt, transurethral resection of the bladder tumor.

Identification of discriminating genes for chemosensitivity. We applied a random permutation test to identify genes that were expressed at a significantly different level between the two groups; that is, tumors with good response and those with poor response to the chemotherapy. Mean (μ) and standard deviation (δ) were calculated from the log-transformed relative expression ratios of each gene in the responder (r) and non-responder (n) cases. A discrimination score (DS) for each gene was defined as follows: $DS = (\mu_r - \mu_n)/(\delta_r + \delta_n)$.

We carried out permutation tests to estimate the ability of these individual genes to distinguish between responders and non-responders; samples were randomly permutated between the two groups, 10,000 times. Since the DS data set of each gene showed a normal distribution, we calculated a P-value for the user-defined grouping (12). For the initial analysis, we applied the expression data obtained for the 18 cases (9 responders and 9 non-responders) that were obtained at an earlier stage of the study.

Table II. List of primer sets and TaqMan probes.

Public ID	Symbol	Forward primer	Reverse primer	TaqMan probe
Internal control				
AF385084	*CCT6A*	5'-CTCCTGCACTGTGATTGCCA-3'	5'-GACATTCCAGCTCGCATGATC-3'	5'-FAM-CAACATTCTCTTGGTTGATG-MGB-3'
Predictive genes				
AL137335	*IPO7*	5'-TTGTGGTGCACTCACCTCTGA-3'	5'-CAATGAAATACCACTAACCCCTTTTT-3'	5'-FAM-AGTGACTTGAATTCGG-MGB-3'
BC043571	*LOC613266*	5'-CCTCCAAGAGTGTTCGATTTCAA-3'	5'-CCTGCGTTCAGGACTGAGTAAGA-3'	5'-FAM-CATTGTGCAATTTC-MGB-3'
BF508662	*SPRY1*	5'-CTTTTGGCCCCTTGGATAGTT-3'	5'-AGGCAAGGAAAACACAGAAGAGA-3'	5'-FAM-ACAGCTGAGTAATTCT-MGB-3'
AI884890	*OSBPL11*	5'-AGGTTCTTCTCTGTTTACCCTAAATCC-3'	5'-CAATCAGGAAGCAGGTCACTCA-3'	5'-FAM-CCCAGAATGGAGTCATT-MGB-3'
NM_016220	*ZNF107*	5'-TGCTCTTCATTCCTATTGTATTCACAT-3'	5'-CATAAATAATACCGACCTAACAGAAATGAT-3'	5'-FAM-CATGCATCAAAGATATGAGA-MGB-3'
AI025829		5'-TGTTTTTTCAGTTGCTGCACTTTTT-3'	5'-GCATATTCCAGCAATTACCTTTGA-3'	5'-FAM-TTTAATCTTGCTCAGTCCC-MGB-3'
AF090916		5'-TGGCAATATCCTTTTCTCTGATTTT-3'	5'-GGCCTTGGTTGCCCAAA-3'	5'-FAM-AAAGTTAGGCTGAGTGCAGT-MGB-3'
N63709	*LIN7C*	5'-CCTCTGCCAACAATCTGGTTTT-3'	5'-CCATACCTGGAATAACCTTTGAAGA-3'	5'-FAM-ATTGTTGTCTAAAGTTTGCTAGTAG-MGB-3'
AL043021	*WDR90*	5'-GCCTGGAGCAAGCTGTTGTAA-3'	5'-CAAAAGGGCAACAGGTATGAAAG-3'	5'-FAM-TTTGGCGCCCTGTGAA-MGB-3'
NM_002555	*SLC22A18*	5'-TTTGGCGTCCCCGTCTT-3'	5'-GGACCAGGAGGACAAGGGTATT-3'	5'-FAM-CACGTGCAGGTTGCTA-MGB-3'
NM_018129	*PNPO*	5'-ATCACACCTGCCTGAGAAGGA-3'	5'-CCTGACGGACTGGGAATAAAAA-3'	5'-FAM-TGGGCTGTCACTAGGA-MGB-3'
NM_005207	*CRKL*	5'-TTGAGGCCATGGCGAGAT-3'	5'-GCAGCTAAGCCACTGCTTTGT-3'	5'-FAM-CTGCATGTTTGCTGTTC-MGB-3'

The probes contain a 6-carboxy-fluorescein phosphoramidite (FAM) label at the 5' end of the gene and a minor groove binder (MGB) and non-fluorescent quencher at the 3' end.

Calculation of the prediction score. We calculated the prediction scores (PS) according to procedures described previously (12). Each gene (gi) votes for either responder or non-responder depending on whether the expression level (x_i) in the sample is closer to the mean expression level of the responders or non-responders in the reference samples. The magnitude of the vote (V_i) reflects the deviation of the expression level in the sample from the average of the two classes: $V_i = l\, x_i - (\mu_r + \mu_n)/2\, l$.

We summed the votes to obtain total votes for the responders (V_r) and non-responders (V_n) and calculated PS values as follows: $PS = [(V_r - V_n)/(V_r + V_n)] \times 100$, reflecting the margin of victory in the direction of either responder or non-responder. PS values range from -100 to 100; a higher absolute value of PS reflects a stronger prediction.

Evaluation of the classification and leave-one-out method. We calculated the classification score (CS) using prediction scores of the responders (PS_r) and non-responders (PS_n) in each gene set as follows: $CS = [\mu(PS_r) - \mu(PS_n)]/[\delta(PS_r) + \delta(PS_n)]$.

A larger value of CS indicates better separation of the two groups by the prediction scoring system. For the leave-one-out test, one sample is withheld, the permutation P-value and mean expression levels are calculated using the remaining samples and the class of the withheld sample is subsequently evaluated by calculating its prediction score. We repeated this procedure for each of the 18 samples.

Quantitative reverse transcription-PCR. Aliquots of the same aRNA hybridized to the microarray from individual samples were reversely transcribed using random hexamer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The expression of the 12 predictive genes and 1 endogenous control gene was measured by quantitative-RT-PCR using TaqMan Gene Expression Assay products on a Light Cycler 480 system (Roche Applied Science, Basel, Switzerland) as described previously (13,14). The sequences of the primers and fluorogenic TaqMan MGB probes are listed in Table II. To normalize the expression of each gene, we selected as internal controls chaperonin-containing TCP1, subunit 6A (*CCT6A*), since this showed the smallest fluctuations of cancer/normal ratio in our bladder cancer microarray data as described previously (13). For the generation of standard curves we used a mixture of mRNAs derived from the bladder cancer samples. Quantitative RT-PCR experiments were performed in duplicate for all 12 predictive genes, and the relative expression ratios of each sample were calculated. The normalized gene expression values were log-transformed (on a base 2 scale) in a manner similar to the transformation of the microarray-based hybridization data.

Simulation of response of GC- or MVAC-treated patients to M-VAC or GC therapy. To simulate the clinical response of GC- or MVAC-treated patients to M-VAC or GC therapy, we first estimated the accuracies of the prediction scoring systems: positive and negative prediction accuracies (PPA and NPA) for test cases with positive and negative prediction scores, respectively. We calculated the accuracy of each of the prediction scoring systems for GC (PPA_{GC}, NPA_{GC}) and

Table III. List of 14 discriminating genes.

Public ID	Gene symbol	Gene name	P-value[a]	Group[b]
AL137335	*IPO7*	Importin 7	$3.96x10^{-7}$	-
BC043571	*LOC613266*	Hypothetical LOC613266	$8.38x10^{-7}$	+
BF508662	*SPRY1*	Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)	$5.34x10^{-6}$	-
AI884890	*OSBPL11*	Oxysterol binding protein-like 11	$1.81x10^{-5}$	+
NM_016220	*ZNF107*	Zinc finger protein 107	$1.96x10^{-5}$	+
AI025829		CDNA clone IMAGE:5287121	$2.45x10^{-5}$	-
AF090916		Clone HQ0312	$2.90x10^{-5}$	+
N63709	*LIN7C*	Lin-7 homolog C (*C. elegans*)	$3.18x10^{-5}$	-
AL043021	*WDR90*	WD repeat domain 90	$3.19x10^{-5}$	+
NM_002555	*SLC22A18*	Solute carrier family 22, member 18	$3.30x10^{-5}$	-
NM_018129	*PNPO*	Pyridoxamine 5'-phosphate oxidase	$4.75x10^{-5}$	-
NM_005207	*CRKL*	V-crk sarcoma virus CT10 oncogene homolog (avian)-like	$6.92x10^{-5}$	+
AL050145		Transcribed locus	$7.78x10^{-5}$	+
BG284709	*LOC283871*	Hypothetical protein LOC283871	$9.11x10^{-5}$	+

[a]P-values were calculated by random permutation tests. [b]+, genes up-regulated in the responders; -, genes up-regulated in the non-responders. Information was retrieved from the Unigene database of the National Center for Biotechnology Information (build #159).

M-VAC (PPA_{MVAC}, NPA_{MVAC}) therapies, respectively. Among the 19 test cases (Fig. 2B) for the GC therapy, all of the 10 cases with positive scores were actually responders. Eight of the 9 cases with negative scores were non-responders. Therefore, the positive and negative predictive accuracies of this system were PPA_{GC} = 10/10 and NPA_{GC} = 8/9. However, in our previous study among the 21 test cases for the prediction scoring system for the M-VAC therapy, 14 of 16 cases with positive prediction scores were responders and the remaining 2 cases were non-responders (data not shown). All of the 5 cases with negative prediction scores were non-responders (data not shown). Therefore, the positive and negative predictive accuracies were PPA_{MVAC} = 14/16 and NPA_{MVAC} = 5/5.

Based on these results, we re-estimated the number of simulated GC/M-VAC responders: the number of estimated non-responders using both the GC and M-VAC prediction scoring systems was, based on the initial 13 cases (Fig. 4A-C), 13 x NPA_{GC} x NPA_{MVAC}. Also, among the 29 cases predicted to be GC non-responders, but M-VAC responders, 29 x NPA_{GC} x (1 - PPA_{MVAC}) cases should be reconsidered as non-responders to both GC and M-VAC. Therefore, of the 76 cases in total, the number of estimated GC/M-VAC responders was calculated to be: 76 - [13 x NPA_{GC} x NPA_{MVAC} + 29 x NPA_{GC} x (1 - PPA_{MVAC})] = 61.2.

Results

Identification of genes associated with GC neoadjuvant chemotherapy for bladder cancer. We enrolled 37 patients with invasive bladder cancer whose clinicopathological features are summarized in Table I. We defined patients who achieved significant tumor shrinking (>60%) after two courses of GC neoadjuvant chemotherapy as 'responders', as we used the definition for the establishment of the prediction score for response to M-VAC therapy (13,15). We compared the microarray expression profiles of tumors from the 9 responders and 9 non-responders and identified a set of genes that distinguished the two groups in accordance with the following criteria: signal intensities higher than the cut-off level in >60% of samples of at least one group. Then, we carried out a random permutation test to select genes that may be associated with the drug response (see Materials and methods). We identified 14 genes that showed permutation P-values of <0.0001 (Table III). As shown in Fig. 1, the expression levels of 8 genes were increased and those of the remaining 6 were decreased in the responder group as compared to the non-responder group. A supervised hierarchical clustering analysis using this set of genes with Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm) yielded good separation of the two groups with regard to the response to GC treatment (Fig. 1).

Establishment of prediction scoring system for clinical response to GC neoadjuvant chemotherapy. Using the 14-gene set that seemed to distinguish the two groups, we calculated the prediction score of each sample by the weighted-vote method (12). Subsequently, we rank-ordered these candidates on the basis of the significance of their permutation P-values (Table III) and calculated prediction scores by the leave-one-out cross-validation test. For the leave-one-out test, we withheld one sample and calculated the permutation P-values and the mean expression levels using the remaining samples to identify genes that most powerfully separated the responder group from the non-responder group. As shown in Table III, importin 7 (*IPO7*) and hypothetical LOC613266 (*LOC613266*) revealed a P-value of $<10^{-6}$ in the permutation test ($P=3.96x10^{-7}$ and $P=8.38x10^{-7}$, respectively). IPO7, which was up-regulated in the tumors belonging to the non-responder group, imports proteins into the nucleus by acting as an adapter-like protein (16) and is reported to mediate the

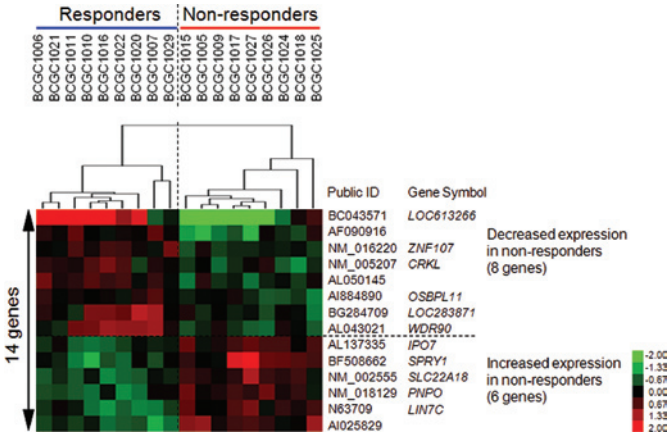

Figure 1. Expression patterns of the 14 genes that discriminated the 9 responders from the 9 non-responders among the bladder cancer patients treated with GC. Horizontal rows represent individual genes; vertical columns represent individual samples. Each cell in the matrix represents the expression level of a single transcript in a single sample, with red and green indicating transcript levels, respectively, above and below the median for that gene across all samples. Black represents unchanged expression or slight expression (intensities of signal under the cutoff value). Color saturation is proportional to the magnitude of the difference.

penetration of the interacting extracellular signal-regulated kinases (ERK) into the nucleus through nuclear pores (16). Since the ERK signaling pathway was reported to be in part responsible for the resistance to gemcitabine in hepatocellular, pancreatic and cholangiocellular carcinomas (17,18), up-regulated expression of IPO7 may contribute to resistance to gemcitabine-combined therapy for bladder cancer through the ERK signaling pathway.

We calculated the classification score (CS) using the prediction scores of the 9 responders and 9 non-responders in various combinations of selected genes and obtained the best separation of the two groups by using the 12 genes that were ranked highest in our candidate gene list (Fig. 2A and B; Table IV). A hierarchical clustering analysis using this set of genes with Cluster and Treeview software yielded good separation of the two groups with regard to sensitivity to the GC neoadjuvant chemotherapy (Fig. 2C).

Furthermore, to verify the prediction scoring system based on the expression data for this set of 12 genes, we examined 19 'test' cases (11 responders and 8 non-responders; Fig. 2B). We investigated gene expression profiles in each of the 19 test cases and then calculated the prediction scores. As shown in Fig. 2B, for 18 of the 19 test cases, the clinical responses were correctly predicted according to the calculated prediction scores. Our data suggest that expression levels of these 12 genes or a subset may play important roles in cellular responses induced by GC neoadjuvant chemotherapy.

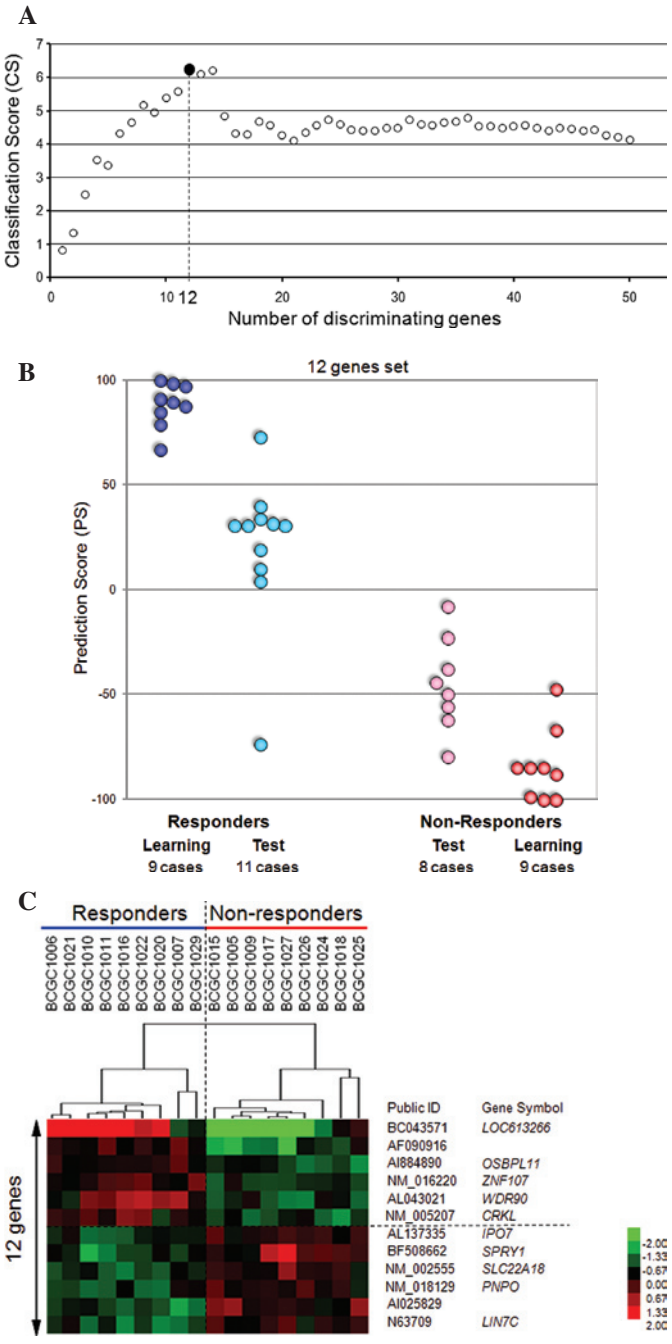

Figure 2. (A) Optimization of the number of discriminating genes. The classification score (CS) was calculated using the prediction scores of the responders (PS_r) and non-responders (PS_n) in each gene set as follows: $CS = [\mu(PS_r) - \mu(PS_n)]/[\delta(PS_r) + \delta(PS_n)]$. A larger value for CS indicates better separation of the two groups by the prediction scoring system. (B) Blue and red circles indicate scores for cross-validation cases of patients whose expression data were used for selecting discriminating genes (learning). Light blue and pink circles represent scores for the 19 additional cases (test). (C) Clustering analysis of 12 predictive genes. All samples fell appropriately into one of two 'trees' according to their sensitivity to GC neoadjuvant chemotherapy.

Establishment of a quantitative reverse transcription-PCR-based prediction scoring system. To further validate the results of the microarray analysis, we carried out real-time quantitative RT-PCR for the 12 predictive genes and one quantitative control gene, *CCT6A*, using the 32 cases (14 learning and 18 test cases) (13). We observed significant concordance between the results from the microarray and those of the quantitative RT-PCR experiments. As shown in Table V, Pearson and Spearman rank correlations were positive and statistically significant for all of the genes.

Hence, we attempted to adapt our prediction system on the basis of quantitative real-time RT-PCR to apply to the clinical test. We performed quantitative real-time RT-PCR of 12 predictive genes for 14 learning and 18 test cases, and calculated the prediction scores for all cases. When we esti-

Table IV. List of the 12 predictive genes.

Public ID	Gene symbol	Gene name
AL137335	*IPO7*	Importin 7
BC043571	*LOC613266*	Hypothetical LOC613266
BF508662	*SPRY1*	Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)
AI884890	*OSBPL11*	Oxysterol binding protein-like 11
NM_016220	*ZNF107*	Zinc finger protein 107
AI025829		CDNA clone IMAGE:5287121
AF090916		Clone HQ0312
N63709	*LIN7C*	Lin-7 homolog C (*C. elegans*)
AL043021	*WDR90*	WD repeat domain 90
NM_002555	*SLC22A18*	Solute carrier family 22, member 18
NM_018129	*PNPO*	Pyridoxamine 5'-phosphate oxidase
NM_005207	*CRKL*	V-crk sarcoma virus CT10 oncogene homolog (avian)-like

Table V. Correlation of microarray expression data with quantitative-PCR-derived values.

Public ID	Gene symbol	Pearson's correlation coefficient	P-value	Spearman's rank correlation	P-value
AL137335	*IPO7*	0.67	$2.6x10^{-5}$	0.69	$2.4x10^{-5}$
BC043571	*LOC613266*	0.88	$6.9x10^{-9}$	0.96	$2.5x10^{-6}$
BF508662	*SPRY1*	0.85	$9.4x10^{-10}$	0.86	$2.7x10^{-7}$
AI884890	*OSBPL11*	0.73	$2.7x10^{-6}$	0.74	$2.9x10^{-6}$
NM_016220	*ZNF107*	0.82	$1.0x10^{-8}$	0.78	$1.1x10^{-6}$
AI025829		0.47	$6.2x10^{-3}$	0.50	$4.0x10^{-3}$
AF090916		0.84	$2.0x10^{-9}$	0.86	$2.5x10^{-7}$
N63709	*LIN7C*	0.81	$1.9x10^{-8}$	0.74	$3.1x10^{-6}$
AL043021	*WDR90*	0.79	$7.1x10^{-8}$	0.79	$9.1x10^{-7}$
NM_002555	*SLC22A18*	0.85	$8.1x10^{-10}$	0.84	$4.3x10^{-7}$
NM_018129	*PNPO*	0.70	$7.4x10^{-6}$	0.70	$1.7x10^{-5}$
NM_005207	*CRKL*	0.54	$1.3x10^{-3}$	0.60	$3.3x10^{-4}$

mated these scores by the leave-one-out cross-validation test, all learning cases and 17 of the 18 test cases were categorized correctly according to their response to GC neoadjuvant chemotherapy (Fig. 3).

Clinical implication of the two systems that predict the response to GC and M-VAC therapy. We previously reported a system with nearly 90% accuracy to predict the clinical response to the M-VAC regimen, a combination of methotrexate, vinblastine, doxorubicin and cisplatin neoadjuvant chemotherapy, for patients with invasive bladder cancer (13). To investigate how patients treated with GC therapy respond to M-VAC therapy, we simulated the clinical response of the 37 GC-treated patients to the M-VAC therapy using our prediction system for M-VAC neoadjuvant chemotherapy (13). As shown in Fig. 4A, 14 of the 19 cases predicted to be GC responders were suspected to be responders to M-VAC therapy, and 11 of 18 cases predicted to be GC non-responders were suspected to be responders to M-VAC therapy according to our M-VAC prediction scoring system. On the other hand, we applied the scoring system to the GC prediction system indicated above to the 39 M-VAC-treated patients that had been reported previously (13). Seven of the 25 cases predicted to be M-VAC responders were calculated to be responders to GC therapy, and 8 of the 14 cases predicted to be M-VAC non-responders were calculated to be responders to GC therapy (Fig. 4B). The distribution of the cases according to the predicted response to GC or M-VAC therapy is summarized in Fig. 4C. Among the the 76 patients treated with either GC or M-VAC, 21 patients were predicted to be responders of both GC and M-VAC therapies, 13 were predicted to be responders to GC therapy, 29 were predicted to be responders to M-VAC therapy and 13 were likely to be non-responders to both therapies (Fig. 4C). Sixty-three of the 76 patients could be expected to respond to both or either of these two regimens by applying our two prediction systems. However, the positive and negative predictive accuracies of the prediction systems for response to M-VAC (PPA_{MVAC}, NPA_{MVAC}) and GC (PPA_{GC}, NPA_{GC}) were: PPA_{MVAC} = 14/16, NPA_{MVAC} = 5/5 (data not shown), PPA_{GC} = 10/10 and NPA_{GC} = 8/9 (Fig.

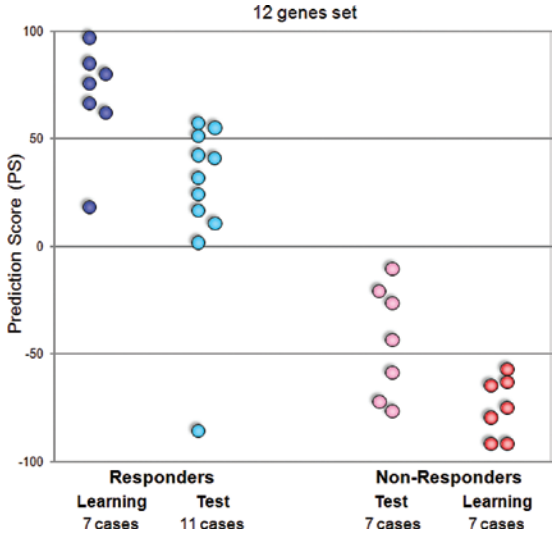

Figure 3. Quantitative RT-PCR-based prediction scoring system. Prediction scores for 32 cases using values derived from quantitative RT-PCR experiments of 12 predictive genes. Blue and red circles indicate scores for selecting discriminating genes (learning cases). Light blue and pink circles represent scores of 18 additional (test) cases.

2B). Hence, 80.1% (1 - [13 x NPA_{GC} x NPA_{MVAC} + 29 x NPA_{GC} x (1 - PPA_{MVAC})]/76) of the 76 patients were calculated to be responders to GC and/or M-VAC therapies (see Materials and methods) by the combination of the two systems. Without these types of prediction system, the responders to GC and M-VAC therapy were limited to 54.1% (20 of 37 cases) and 59.0% (23 of 39 cases), respectively (Fig. 4). Since patients usually are able to undergo neoadjuvant treatment once, the application of these prediction scores should improve the quality of life of cancer patients.

Discussion

Microarray analysis is now widely applied to examine the expression of thousands of genes simultaneously in cancer cells. However, in the great majority of previous reports, adequate attention has not been paid to the quality of the materials and experiments. For example, clinical samples (surgically resected tissues or biopsy materials) usually consist of various cellular components, and the proportions of cancer cells in given individual tissues vary enormously from one tumor to another (19). To obtain precise expression data of cancer cells, we applied a laser microbeam microdissection system to enrich as much as possible the populations of cancer cells from the biopsy specimens of 37 invasive bladder cancers in order to establish a scoring system to predict response to GC therapy.

Despite recent advances, approximately 50% of patients with bladder cancer who receive GC chemotherapy show no or very poor response in terms of staging, and a large proportion of them suffer from adverse events, such as myelosuppression and/or gastrointestinal toxicity (9,10). Although certain factors have been reported to be associated with chemosensitivity or prognosis of bladder cancer patients (20-24), characterization of tumor features using only one or a few of these factors has failed thus far to reliably predict individual responses, indicating a need for a more accurate method for predicting response to anticancer drugs. This study was designed to develop a prediction system for GC neoadjuvant chemotherapy on the basis of gene expression profiles of purified populations of bladder cancer cells. We identified 14 genes whose expression was significantly different between the responders and non-responders and further ranked them by statistical significance of the permutation test (P<0.0001; Table III). Then, we further selected 12 genes and established the numerical scoring system. Moreover, we tested the scoring

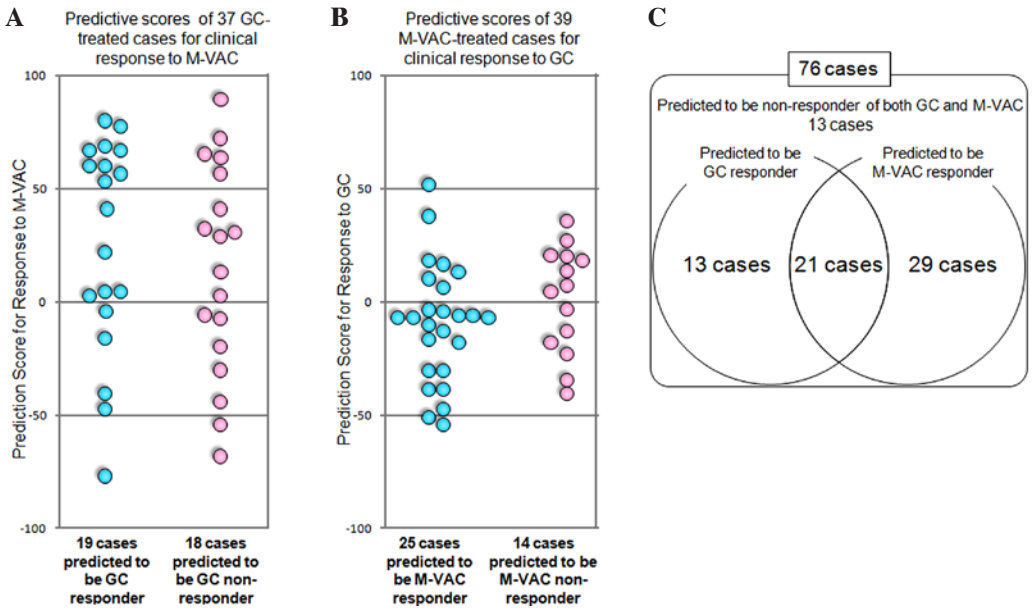

Figure 4. (A) Distribution of the prediction scores of 37 GC-treated patients for clinical response to M-VAC chemotherapy. Light blue and pink circles represent cases predicted to be GC responders and non-responders, respectively. (B) Distribution of prediction scores of 39 M-VAC-treated patients for clinical response to GC chemotherapy. Light blue and pink circles represent cases predicted to be M-VAC responders and non-responders, respectively. (C) Venn diagram indicating the distribution of the 76 cases according to the response to GC or M-VAC therapy.

system by the leave-one-out cross-validation method and found it to provide the best separation of the responders from the non-responders. Furthermore, our scoring system was able to predict accurately the response of 18 of the 19 additional test cases to GC neoadjuvant chemotherapy (Fig. 2B).

The list of 14 genes that showed significant differences between the two groups might provide insight into the biological mechanism(s) underlying sensitivity or resistance to GC chemotherapy. Among those 14 genes, *IPO7*, which imports protein into the nucleus by acting as an adapter-like protein, was up-regulated in the non-responders (Fig. 1, Table III). Although hypoxia inducible factor 1, α subunit (HIF-1α), which is one of the substrates of IPO7, is known to activate the connective tissue growth factor/cysteine-rich 61 (CCN1) (25), CCN1 was found to confer resistant to carboplatin or cisplatin-induced apoptosis by inhibiting caspase-3 activity on ovarian cancer cells (25,26). Therefore, up-regulated expression of IPO7 may contribute to resistance to GC neoadjuvant chemotherapy through inhibiting caspase-3 activity. Furthermore, solute carrier family 22, member 18 (*SLC22A18*), which is a polyspecific organic cation transporter, was up-regulated in the non-responders. This gene encodes a predicted protein with multiple membrane-spanning segments that belongs to the polyspecific transporter/multi-drug resistance gene family (27). The pharmacogenomic approach based on the correlation of expression and sensitivity data sets derived from the NCI-60 cell panel suggest that SLC22A18 could be a transporter of gemcitabine (28). Hence, up-regulated expression of SLC22A18 may contribute to resistance to GC neoadjuvant chemotherapy by the efflux of gemcitabine from cancer cells through the transporter.

Previously, other research groups have predicted the prognosis or chemosensitivity of tumors based on quantitive RT-PCR results for the expression of genes selected through microarray analysis (29,30). To confirm the reliability of microarray data and open the possibility of more convenient prediction strategies for routine clinical use, we also performed quantitive RT-PCR experiments using the 12 predictive genes and the 32 learning and test cases of bladder cancer selected after microarray analysis. We confirmed a significant correlation between the data obtained for the 32 paired samples upon microarray and results of quantitive RT-PCR (Table V). Moreover, we verified that our quantitative RT-PCR-based prediction system could also correctly classify 17 of the 18 subsequent test cases with regard to their drug response (Fig. 3).

We previously reported a scoring system to predict response to M-VAC therapy, applying a laser microbeam microdissection system (13). In the present study, in order to simulate how patients treated with GC or M-VAC therapy respond to M-VAC or GC therapy, we calculated their prediction scores for the response to M-VAC or GC therapy using the two prediction systems, respectively. As shown in Fig. 4, among the 76 patients, only 21 and 13 cases were expected to be responders and non-responders to both GC and M-VAC therapies, respectively, suggesting that the sensitivity or resistance to M-VAC therapy was unlikely to be correlated with the sensitivity or resistance to GC therapy (Fig. 4C). Although a number of patients with invasive bladder cancer have received neoadjuvant chemotherapies, such as M-VAC or GC without prediction of their responses, the response rate (complete or partial response) to either of the GC or M-VAC therapy has been reported to be approximately 50% (7-10). Since our present and previous studies indicated that positive predictive accuracies for M-VAC and GC were 87.5% (14/16; data not shown) and 100% (10/10), respectively, personalized selection of an appropriate chemotherapy with a combination of the two prediction systems would be expected to improve the response rate to the chemotherapies. In addition, if all patients with invasive bladder cancer were treated with appropriate therapy based on the results of prediction systems, 80.1% would achieve significant tumor shrinking (>60%) by either or both of the two regimens.

In conclusion, we imply with some confidence that our prediction system for the sensitivity of invasive bladder cancers to GC therapy as well as M-VAC therapy, which was reported previously on the basis of either the microarray-derived expression profiles or the quantitative RT-PCR results, should provide opportunities for achieving better prognosis and improved quality of life for patients, leading to higher response rate of neoadjuvant chemotherapy, although a larger scale study is certainly warranted. Moreover, appropriate neoadjuvant chemotherapy for each patient with invasive bladder cancer using these systems might encourage minimal surgery for invasive bladder cancer. Our data suggest that the goal of 'personalized medicine', prescribing the correct treatment regimen for each patient, may be achievable by selecting specific sets of genes for their predictive values according to the approach demonstrated here.

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