

Prediction of doxorubicin sensitivity in gastric cancers based on a set of novel markers

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Abstract. Chemotherapy is the standard treatment for patients with advanced gastric cancer; however, it has been difficult to predict chemotherapy response. In the current study, we attempted to develop a prediction model for individual response to doxorubicin chemotherapy in gastric cancer patients based on the hypothesis that expression analysis of a set of key drug sensitivity genes for doxorubicin could allow us to predict therapeutic response. From literature and our previous microarray data, the genes correlative in the expression levels with doxorubicin response were chosen. We selected seven reliable prediction markers for doxorubicin from 90 candidate sequences. Using expression data of genes quantified by real-time reverse transcription-PCR in 20 specimens, we fixed a linear model by multiple regressions, which converted the quantified expression data into a calculated inhibition rate of doxorubicin. Using the same set of genes, we then validated the formula in an independent set of 19 specimens. Our results suggest that the response of gastric cancer to doxorubicin can be predicted by expression patterns in this set of genes. The response prediction model will be of practical use to evaluate patient before chemotherapy.

Introduction

Gastric cancer ranks as the second leading cause of cancer-related mortality worldwide (1). With early diagnosis,

gastrectomy with lymph node dissection results in a >90% 5-year survival rate, however, many cases are considered non-resectable at diagnosis because of locally advanced or metastatic disease. Chemotherapy then becomes one of the few treatment options. Doxorubicin and its derivatives are widely used in various chemotherapy regimens in combination with other chemicals. In a perspective study, a regimen including epirubicin, cisplatin and fluorouracil improved the 5-year survival rate among patients with advanced gastric adenocarcinoma from 23 to 36% (2). However, these regimens are not entirely satisfactory because mean average survival can only be extended ~6 months compared to best supportive care only (3). This poor therapy efficacy may be due to the fact that many gastric cancers are naturally resistant to many anticancer drugs, or acquire resistance during prolonged treatment. Therefore, predicting the occurrence of drug resistance becomes a major topic for successful chemotherapy of gastric cancers, unfortunately, to date, clinical tests for predicting cancer chemotherapy response are not available. Selecting an optimal regimen for each individual based on gene expression profile, or so-called personalized medicine has been proposed (4,5). However, individual drug resistance related markers including P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) have shown little predictive value (6).

Recently, using multiple genes or even microarray data to predict chemosensitivity has achieved some success in certain types of tumors though still lacks support from a large scaled clinical trial (7-11). However, various genetic events are involved in the mechanisms of drug sensitivity in different tumor types to different chemotherapy drugs, it would be important to know which gene set could be useful in predicting the doxorubicin response of gastric cancer in chemotherapy. In the current study, we selected multiple candidate genes based on previous gene profiling data, then a prediction model of doxorubicin response was developed by multiple regression analysis using selected genes and verified in gastric cancer specimens.

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Table I. The primer sets.

Gene symbol		Primer sequence	Position	Product length
CYR61	F	5'-CCAGAAATGTATTGTTCAAAC-3'	886	242
	R	5'-TCACACTCAAACATCCAG-3'	1,127	
GNAI1	F	5'-CCAGAGATTCAAAACCCCAAACC-3'	72	83
	R	5'-GCGGCTGTTCCCTCACGAC-3'	154	
IFITM1	F	5'-AAACAGCAGGAAATAGAAAC-3'	1	163
	R	5'-GGAAGTGTGAGTGAAGG-3'	163	
G1P2 ^a	F	5'-TGGTGGACAAATGCGACGAA-3'	296	242
	R	5'-CAGGCGCAGATTCATGAACA-3'	537	
ADAM22	F	5'-TTGCTGTCCTCTGAATAC-3'	407	217
	R	5'-AATCCTCTTGAGTAGTGTC-3'	623	
SPHK1	F	5'-CTCTGGTGGTCATGTCTG-3'	935	152
	R	5'-AGCATAATGGTTCAAGGAAG-3'	1,086	
FN1	F	5'-TAACTGCGAGAGTAAACC-3'	521	203
	R	5'-TGTCACCAATCTTGTAGG-3'	723	

^aAll primers were designed using AlleleID v5.01 software (Premier Biosoft International, Palo Alto, CA) except for G1P2, which was obtained from <http://www.realtimeprimers.org/>.

Materials and methods

Patients. Human specimens from surgically resected gastric cancers and corresponding clinical information were collected from three hospitals (Fourth Military Medical University, Southern Medical University and Qingdao University). A total of 45 samples (10 women and 25 men; median age, 65; range, 37-82 years) were selected for the study. All of the patients had histologically proven gastric cancer and had not received any treatment before tumor sampling. The patients were required to have no significant baseline laboratory abnormalities with performance status (WHO) 0-2. The study protocol was approved by the Human Ethics Review Committee of the Fourth Military Medical University and informed consent was obtained from the patients before the start of this study. The tumor specimens were randomly divided into two groups: training group and validation groups. The former was used as experimental samples to develop a prediction model and the latter was used to test samples to confirm the predictive accuracy of the developed model. Samples were cut into three pieces: one piece was HE-stained to histologically confirm the proportions of cancer cells in the specimens >70%, one was subjected for histoculture drug response assay (HDRA) and one was stored at -80°C until real-time PCR analysis.

Histoculture drug response assay. HDRA was carried out following the protocol published previously (12). Briefly, the specimens were scissor-minced into pieces ~0.5-mm in diameter, which were then placed on each of the prepared collagen surfaces in 24-well plates. The plates were incubated for 7 days at 37°C with 15 µg/ml doxorubicin (Sigma, St. Louis, MO) dissolved in RPMI-1640 medium containing 20% FCS in a humidified atmosphere containing 95% air and 5% CO₂. After histoculture, 100 µl HBSS

containing 0.1 mg/ml collagenase (type I, Sigma) and 100 µl 5 mg/ml MTT (Sigma) solution were added to each well and incubated for another 8 h. After extraction with DMSO, the absorbance of the solution in each well was read at 540 nm. The absorbance/g of histocultured tumor tissue was calculated from the mean absorbance of tissue from four culture wells and tumor-tissue weight determined prior to culture. The inhibition rate was calculated using the formula: Inhibition rate (%) = (1-mean absorbance of treated tumor/g/mean absorbance of control tumor/g) x 100.

cDNA preparation. RNA isolation and reverse transcription was performed within one week of storage at -80°C. The smashed tissue samples were lysed with TRIzol Reagent (Invitrogen, Carlsbad, CA) and RNA was isolated following the manufacturer's protocol. RNA quality from each sample was assessed by visualization of the 28S/18S ribosomal RNA ratio. Total RNA (2 µg) extracted from tumor specimens was reverse transcribed using random hexamer and Superscript II (Invitrogen) according to manufacturer's protocol.

Real-time reverse transcription-PCR. Aliquot of the cDNA (equivalent to 10 ng total RNA) was subjected to real-time reverse transcription-PCR (RT-PCR). The primer sets are supplied in Table I. Each reaction was carried out in triplicate using a DNA Engine Opticon2 system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Briefly, the PCR reaction was carried out in a 10 µl final volume containing the following: 5 µl 2 X SYBR-Green I master mix (Qiagen, Valencia, CA), 0.5 µl 10 µM forward primer and reverse primer; and 1.0 µl diluted cDNA and 3 µl water. After an initial denaturation step at 95°C for 5 min, temperature cycling was initiated. Each cycle consisted of denaturation at 94°C for 10 sec, annealing at 55°C for 15 sec and elongation at 72°C for 15 sec. A total of 40 cycles were performed. These

Gene symbol	Accession	Gene symbol	Accession	Gene symbol	Accession
ABCB1	AA135957	ERBB3	AA877334	OLR1	AA682386
ACAT2	R25823	FLJ12953	R39223	PALM2-AKAP2	AA488418
ADAM22	AA461474	FLJ31340	AA644099	PAX6	R95962
	N29801	FN1	AA953560	PCOLCE	AA513387
ALDH1B1	AA702358		N73836		AA670200
ARFGEF2	AA099582	G1P2	AA120862	PELI1	W86504
	AI961669	GBP1	AA280278	PHTF2	AA284466
ASK	AI952661		AA486849		AI017873
BEAN	R11384	GBP2	W01896		AI095488
BF	AA401441		W72748		AI821373
C19orf32	AA443936	GNAI1	AI140462		R14908
C2orf33	W74362		AW072795	PJA1	AA779217
C7orf23	W48646		W46769	PLAC8	AA150254
CDH5	T53625	GSS	AA463458	POLG2	AI023804
CES2	AI955681	HABP4	AA425398	PPGB	AA916327
CLECSF2	H11732	HLA-E	R94660	RAD21	AA683102
COL5A2	AA599273		AA703079	RG9MTD2	AA987337
	AA857098		AA988615	SDBCAG84	AA457092
CROT	AA043550	HMG20B	AA775743		AI961469
	W84716	HSD17B2	AA970760	SH3KBP1	AA989257
CRYZ	R13434	IFITM1	AA058323	SPHK1	AI341901
CYR61	AA012892	ITPR1	AA035450	SRI	H60859
DDX58	N29630	LEPREL1	AA894672	STCH	H85311
DHRS8	AI220736		AA894672	STX11	AA884281
DMTF1	AA129860	MCFP	AA465188	TFRC	AA677486
DOCK11	AA490315	MGC2574	AA777656		N21329
DPP9	AA007308	NCL	AA433818	TGFB2	AA176249
	AA011400	NOD27	AI016022	TNFAIP3	AA053239
EPLIN	AA487557	NPC1	AA634267	TPBG	AA425666
EPS8L3	AA134985	NPC2	AA630449	UBR1	AA665730

triplicate measurements were averaged and relative gene expression levels were calculated as a ratio to β -actin expression level.

Statistics. For identifying prediction candidate genes, the PAM package v.2.1 for EXCEL was used, as described previously (13). Multiple regression analysis was carried out by NLReg software v.6.2 (Brentwood, TN). Statistical analyses comparing two groups were performed using SPSS11.0 software (Chicago, IL). Probability of <0.05 was considered statistically significant.

Results

Identifying prediction gene candidates from microarray data. To identify discriminatory genes for predicting sensitivity to doxorubicin, we obtained two sets of gene expression profiles of closely related drug-resistant and drug-sensitive gastric cancer cell lines. The EPG-257P and its doxorubicin-resistant

variant EPG-257RDB described by Lage and Gyorffy was obtained from the Stanford Microarray Database (<http://genome-www5.stanford.edu>) (7,14). Microarray data for our previously established SGC7901 and SGC-7901/ADR was from unpublished results using a human genome 70-mer oligonucleotide microarray (CapitalBio Corporation, Beijing, P.R. China) (15). We compared the expression patterns of the doxorubicin-resistant cell lines to the parental cell lines. Since the RNA from EPG-257RDB was prepared from daunorubicin treated cells and SGC-7901/ADR from untreated cells, the significant changes were defined as 5- and 2-fold, respectively. In all, 10144 and 471 sequences were found to be differentially expressed (data not shown). To select discriminatory genes, we compared these two sets of resistance-associated genes. Ninety sequences were found to have similar changes in both sets, among them, all except one gene (PJA-1) was upregulated in resistant cells (Table II).

To select the most potent prediction marker genes from such a large number of candidates, we further verified this set

Table III. Selected prediction marker gene candidates.

Gene symbol	Gene name	Ref Seq	Locuslink ID	EPG-257: EPG-257RDB ratio	SGC-7901: SGC-7901/ADR ratio
ADAM22	A disintegrin and metalloproteinase domain 22	NM_016351 NM_021722 NM_021723	53616	0.1878	0.0823
CYR61	Cysteine-rich, angiogenic inducer, 61	NM_001554	3491	0.1311	0.4952
IFITM1	Interferon induced transmembrane protein 1 (9-27)	NM_003641	8519	0.1850	0.4617
FN1	Fibronectin 1	NM_212474 NM_212475 NM_212482 NM_212476 NM_002026 NM_212478	2335	0.1089	0.1984
SPHK1	Sphingosine kinase 1	NM_182965 NM_021972	8877	0.0753	0.3314
G1P2	Interferon, α -inducible protein (clone IFI-15K)	NM_005101	9636	0.1421	0.3326
GNAI1	Guanine nucleotide binding protein (G protein), α inhibiting activity polypeptide 1	NM_002069	2770	0.1699	0.2334

of genes using the expression profiles of a previously defined group of clinical specimens of gastric cancer described by Chen *et al* (16). The data were obtained also from the Stanford Microarray Database. Only patients at stage III and IV were included in the evaluation since chemotherapy regimens containing doxorubicin were most common treatment options only for advanced gastric cancer patients and the staging factor has been well known as an independent prediction factor for survival. Among these patients, 15 with the longest survival time were regarded as chemotherapy sensitive cases, while 16 with the shortest survival time were regarded as resistant ones. The Prediction Analysis for Microarrays (PAM) training analysis was performed at thresholds 0.9. Centroid plots for the top seven genes associated with doxorubicin sensitivity are shown in Fig. 1 and Table III. Though ABCB1 has been shown to be overexpressed in both the study of Gyorffy *et al* and our data, it fails to produce a significant correlation with this set of clinical data.

Developing a doxorubicin response prediction model on gastric cancer specimens. The doxorubicin response was obtained using Histoculture Drug Response Assay (Table IV) (12). In 23 gastric cancer specimens, 20 produced evaluable results, three cases were eliminated due to contamination or

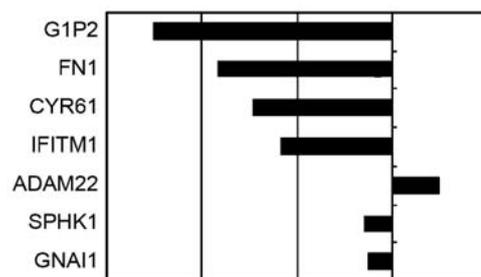


Figure 1. Centroid plot showing the top seven clones with highest potential on doxorubicin sensitive groups after shrinkage with threshold 0.8. Gene names are shown.

low absorbance of extracted formazan of the control tumor. The seven selected genes were subjected to real-time RT-PCR analysis for quantified expression on 20 gastric cancer specimens in training group to confirm correlation with doxorubicin response. Using expression data of selected seven candidate genes quantified by real-time RT-PCR, we performed multiple regression analysis using NLReg software to compose prediction models for the *in vitro* activity of doxorubicin. As shown in Table IV and Fig. 2, the



Clinical and pathological factors	Training group (n=20)	Validation group (n=19)
Age ^a	61.45±11.58	63.32±13.59
Sex ^b		
Male	11	12
Female	9	7
Stage ^b		
I+II	6	9
III+IV	14	10
Tumor site ^b		
Antrum	13	9
Cardia	4	7
Body	3	3
Histology ^b		
Intestinal	14	15
Diffused	2	3
Mixed	4	2
Doxorubicin response ^c	34.65 (12.7-89.2)	32.50 (15.0-80.3)

^aData are shown as mean ± SD. There was no significant difference between the training and validation groups. ^bData are shown as number of cases. Not significant by χ^2 test. ^cData are shown as median (range). Not significant by Mann-Whitney's U test.

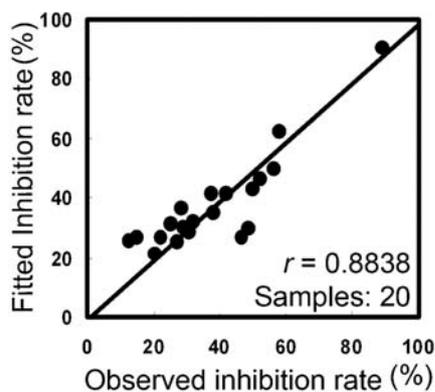


Figure 2. Prediction model for *in vitro* doxorubicin response. Using multiple regression analysis, we developed formulae to predict the response to doxorubicin using the variable expression data of selected marker genes. $\text{Ln}[\text{inhibition rate}] = 3.967 * \text{Ln}[\text{ADAM22}] - 3.981 * \text{Ln}[\text{CYR61}] + 0.453 * \text{Ln}[\text{FN1}] - 1.459 * \text{Ln}[\text{SPHK1}] + 0.983 * \text{Ln}[\text{GNI1}] + 3.076$ (gene names inside brackets indicate the expression level of indicated genes).

observed correlation coefficient indicated potent predictive values of the fixed formula. The NLReg analysis provides estimated θp with P, where a lower P indicates a lower probability for the observation that θp could be 0 in the formula. A positive θ indicates that the corresponding explanatory variable acts as a positive factor in the formula, while a negative one indicates the inverse. The levels of θ do not directly account for the importance of the variable. IFITM1 and G1P2 were eliminated from the final formula since the estimated P for each θp was much higher than those of the others.

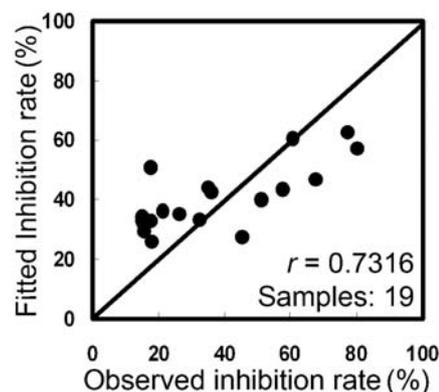


Figure 3. Validation of the prediction model for *in vitro* doxorubicin response. Using an independent set of data, the prediction model was validated in 19 gastric cancer specimens.

Validation of prediction model. To confirm the prediction accuracy of the fixed formula, the prediction model was validated in a validation set of gastric cancer specimens in the same way using the same genes. Twenty-two cases of gastric cancer specimens were collected. Three samples were eliminated and the 19 cases left were subjected to real-time RT-PCR analysis to quantify the expression levels of five selected marker genes and doxorubicin response was also tested using Histoculture Drug Response Assay (Table IV). Despite of the limited number of samples, the results showed that the current prediction model reliably predicted the response of cancer specimens to doxorubicin ($r=0.73$, Fig. 3).

Discussion

In this study, we selected seven marker genes by comparing microarray data from drug-resistant cell lines and subsequent PAM analysis, then determined expression data of the selected genes by real-time RT-PCR. By examining the variable expression levels of the component genes, the efficacy of doxorubicin was predicted using multiple regression analysis. Two more marker genes were eliminated at following expression data regression analysis. To avoid the possible artificial bias, we further validated the model in an independent set of samples. Although the functional significance of these novel markers in drug sensitivity is poorly understood, their expression levels were shown to be correlative with cellular sensitivity to doxorubicin *in vitro*.

Our study provided five novel genes for doxorubicin response prediction, including ADAM22, CYR61, FN1, SPHK1 and GNAI1. Among them, ADAM22 is a membrane-anchored protein involved in cell-cell and cell-matrix interactions (17). CYR61 encodes a secreted, cysteine-rich, heparin-binding protein promoting the adhesion of endothelial cells through integrin and augments growth factor-induced DNA synthesis (18). FN1 is a glycoprotein involved in cell adhesion and migration process (19). SPHK1, a kinase that catalyzes the phosphorylation of sphingosine, may be necessary for the maintenance of tumor cell growth (20). GNAI1 is an α -subunit of heterotrimeric G protein, which is involved as modulator in various transmembrane signaling systems (21). Their functions remain largely unknown, but various results suggest their possible roles in drug sensitivity: FN1 has been implied to be a potent predictor for platinum resistance in ovarian cancer (22). SPHK1 (sphingosine kinase) was reported to be overexpress in chemotherapy-resistant prostate cancer and leukemia cell line, and may be involved in regulating the sensitivity of Dictyostelium discoideum cells to cisplatin (23-28). Cells expressing sphingosin-1-phosphate lyase showed an increase in sensitivity to doxorubicin, which supports our findings (26).

In our study, several previously well-known drug-resistance genes including ABCB1 failed to be selected as one of predictors possibly due to the fact that ABCB1 is more likely to achieve overexpression in patients with multiple chemotherapies. Di *et al* reported that tumors acquired rapid increase in the expression of ABCB1 within days of the start of treatment (29). We have not verified this prediction model in patients with multiple chemotherapies. Current enrolled patients will be continually followed up to achieve data after multiple chemotherapies.

This is the first attempt to use expression profiling data for doxorubicin response prediction in fresh gastric cancer samples. Tanaka *et al* proposed that ABCB1 and TOP2A as doxorubicin sensitivity prediction factors, however these two genes have only been tested in cultured cells (8). Gyorffy *et al* also tried to link gene expression levels to doxorubicin responses with chemosensitivity in breast cancer (7). However, the proposed 79 genes associated with doxorubicin resistance did not overlap with our five suggested predictors, possibly because of the dramatic difference in genetic background between breast and stomach. In gastric cancer,

progress on the prediction model of 5-fluorouracil has been achieved recently using microarray data. A 'Response index' system has been proposed by Matsuyama *et al*, which consists of TNFRSF1B, SLC35F5 and OPRT (30). However, the main obstacle lies in that many studies were restricted within cultured cell lines or limited patient cases (31), the significance of such prediction models need to be further verified on a larger scale.

Recently, much attention has been focused on microarray as a tool of the molecular prediction of drug response and survival. Profiling of gene expression patterns on genome-wide microarrays enables investigators to perform comprehensive analyses of abnormal molecular events in cancer cells. Several hopeful results have been published in various tumor types including leukemia, and ovarian cancer (7,32-34), however, due to the high cost of microarray and uncertain importance to patients survival, models with a few sensitivity predictors selected on microarrays might be more applicable in clinical practice (11,35,36). Our prediction model may have some advantages in the prediction of drug response, such as low cost, easy to standardize and short time duration. However, the key issue to determine the possible clinical application lies in the prediction accuracy. This will call for further validation on a larger scale.

Various methods have been used to test the *in vitro* sensitivity of chemotherapy drugs (37,38). We chose to use HDRA due to the fact that the HDRA allows tumor cells to maintain their native three-dimensional tissue architecture and viability longer than the cell suspension assay. Chemosensitivity determined by HDRA correlated well to clinical response (39). In our study, the HDRA demonstrated a high rate of evaluability (86.7%). However, overall sensitivity of doxorubicin by HDRA is not satisfactory in gastric cancer specimens, ~30.4% (11/39) in our study. The poor response rate is consistent with those previously reported (12). Giving the fact that doxorubicin still remains a common therapy option in chemotherapy regimens of gastric cancer patients; the low response rate of gastric cancer to doxorubicin should call the attention of clinicians. The response prediction model will be of practical use to evaluate the patient before chemotherapy.

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