CXCR4, A Potential Predictive Marker for Docetaxel Sensitivity in Gastric Cancer

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Abstract. *The aim of this study was to explore predictive genes involved in docetaxel sensitivity of gastric cancer. Materials and Methods: Microarray analysis was performed to explore various gene expression levels between parental and docetaxel-resistant cells. A panel of 11 genes selected according to microarray analysis were validated and tested further in 11 cancer cell lines, resulting in 4 genes, CXCR4, CDK6, USP15 and CDH1. Histoculture drug response assay (HDRA) was used to examine docetaxel sensitivity, while qRT-PCR was used to measure the mRNA levels of the genes in 25 surgically dissected gastric cancer specimens. Results: Only CXCR4 mRNA levels in gastric cancer tissues were correlated with docetaxel sensitivity (R^2=0.23, p=0.019) and significantly higher in resistant specimens (p=0.038). AMD3100, a CXCR4 antagonist, enhanced the docetaxel cytotoxicity in vitro. Conclusion: CXCR4 mRNA expression levels may be a potential predictive biomarker in gastric cancer.*

Chemotherapy is an indispensable element of treatment for gastric cancer patients, and fluoropyrimidines, platinum-containing agents, taxanes, alone or in combinations, are the most effective and commonly used chemotherapy regimens (1). Docetaxel is among the second generation of taxanes, demonstrating a stronger anticancer effect than paclitaxel with regard to the promotion of tubulin polymerisation and inhibition of depolymerisation (2). It results in the arrested growth of tumour cells at G_2-M phase in a variety of tumours, including non-small cell lung cancer (NSCLC), hormone-refractory prostate cancer, advanced gastric cancer and breast cancer (2). Although docetaxel is arguably among the most effective chemotherapeutic agents available today, intrinsic and acquired drug resistance remain the main obstacles for its anticancer efficacy and clinical application. In gastric cancer, the efficacy of docetaxel combined with cisplatin/ fluorouracil remains unsatisfactory, with a clinical response rate of 37%, and some patients suffer form adverse effects without any benefit (3).

Personalised chemotherapy based on pharmacogenetics and pharmacogenomics has shown a promising predictive role to achieve superior outcomes in cancer treatment (4-7). Considerable effort has been made to explore predictive markers, especially for use of 5-fluorouracil (5-FU) and platinum, in contrast to a paucity of research focused on taxanes. Class III β-tubulin (TUBB3), microtubule-associated protein tau (MAPT) and breast cancer 1 early onset (BRCA1) are the most widely studied predictive biomarkers for taxane treatment (8-10). However, most of the relevant studies have been somewhat controversial and the predictive accuracy of these genes is still limited (11, 12).

The present study established a docetaxel-resistant gastric cancer cell line and evaluated the alteration of mRNA expression levels globally by microarray, in an attempt to obtain novel predictive biomarkers for docetaxel chemotherapy.

Materials and Methods

Reagents and cell lines. Docetaxel (Doc) was donated by Jiangsu Hengrui Medicine Company (Jiangsu, China, purity >99.9%). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and AMD3100 were obtained from Sigma Chemical Company (St. Louis, MO, USA). All chemicals used in this study were of the highest purety available. Human gastric cancer cell lines BGC-823 and SGC-7901, oesophageal cancer cell line CaEs-17, hepatocellular carcinoma cell lines SMMC-7721, QGY-7701 and HepG2, pancreatic cancer cell line Panc-1 and colon cancer cell lines LoVo and Ls174T were obtained from the Shanghai Institute of Cell Biology (Shanghai, China). Human

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gastric cancer cell lines MKN-45, Kato Ⅲ and AGS were preserved in our laboratory. Cells were cultured in RPMI-1640 medium (Gibco, USA) with 10% fetal bovine serum at 37˚C with 5% CO_2. Doc-resistant cancer cell line BGC-823/Doc was developed as described previously (13).

Cell cycle analysis. Analysis of the cell cycle was performed using a Cycle Test plus DNA reagent kit (Becton Dickinson, CA, USA). Briefly, cells were seeded and treated with Doc for 48 h and 72 h, respectively. They were then harvested and washed twice. After being stained with 25 μg/ml propidium iodide solution, cells were analysed using a flow cytometry (Becton Dickinson, CA, USA).

Oligonucleotide microarray study. Microarray analysis was performed according to Affymetrix protocols (Santa Clara, CA USA). In brief, poly (A)+ mRNA of the parental and resistant cells was extracted with a poly(A)+ mRNA extraction kit (Qiagen, Germany). cRNA was synthesised using the GeneChip 3'-Amplification Reagents One-Cycle cRNA Synthesis Kit (Affymetrix, USA). The labeled cRNAs were then purified and used for construction of the probes. Hybridisation was performed using the Affymetrix GeneChip HG-U133 Plus 2.0 array for 16 h at 45˚C. Signal intensities were measured using a GeneChip Scanner3000 (Affymetrix, USA) and converted to numerical data using the GeneChip Operating Software, version 1 (Affymetrix, USA).

Histoculture drug resistance assay (HDRA). The chemosensitivity test was approved by the Ethics Review Board of Drum Tower Hospital. All samples were collected from the Department of General Surgery of this hospital from December 12, 2007 to June 13, 2008. HDRA was performed according to the protocol of Hoffman *et al*. (14) with slight modification. Briefly, tissue samples were collected and minced using scissors into pieces of around 10 mg. The minced tissues were placed on the top of gelatin sponges prepared by immersing in RPMI-1640 medium supplemented with 20% foetal bovine serum. The test group (n=4) contained 100 μg/ml Doc, while the control group (n=4) did not contain chemotherapeutic reagents. After incubation for 7 days at 37˚C with 5% CO_2, a mixed solution of 100 μl of 1 mg/ml collagenase I and 100 μl of 5 mg/ml MTT with 100 mg/ml sodium succinate was added. After extracting the formazan with DMSO, absorbance (OD) was determined at 490 nm with 630 nm taken as a reference. Inhibition rates (IR) were calculated as:

$$IR\ (\%)=[1-(OD_{test}/Weight_{test})/(OD_{con}/Weight_{con})] \times 100\%.$$

RNA extraction and quantitative RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesised using a Primescript reverse transcription kit (Takara, Japan). Quantitative PCRs were performed using SYBR GREEN I qPCR kit (Toyobo, Japan) and a fluorescent temperature cycler (Mx3000P; Stratagene, USA). Primer sequences are shown in Supplementary Table III. PCR conditions were 95˚C for 1 min, followed by 45 cycles at 95˚C for 15 s, then 60˚C for 1 min. Each reaction was carried out in triplicate.

Statistical analyses. Pearson and Spearman correlation analyses were used to analyse the relations between various gene expression levels and IC_{50}s of 11 cell lines. Fisher's exact test was performed to determine the difference according to patient characteristics. The Mann-Whitney *U*-test was used to compare the sensitive and resistant gastric cancer specimens in terms of their relative gene expressions. All statistical analyses were conducted with SPSS version 13.1 (SPSS Inc, Chicago, IL, USA). A value of $p<0.05$ was considered significant, and all tests were two-tailed.

Table I. *Fold changes of selected gene levels in resistant and parental cell lines*.

Genes	Fold change		Chromosomal location	RefSeq Transcript ID
	Microarray	Q-PCR		
DCN	0.3078	0.4830	chr12q21.33	NM_001920 /// NM_133503 /// NM_133504/// NM_133505 /// NM_133506 /// NM_133507
APC	34.2968	6.4980	chr5q21-q22	NM_000038
CDH1	0.2333	0.4033	chr16q22.1	NM_004360
CXCR4	0.3789	0.4322	chr2q21	NM_001008540 /// NM_003467
PIK3CA	2.4623	6.0210	chr3q26.3	NM_006218
CCL5	13.9288	114.5632	chr17q11.2-q12	NM_002985
SMC3	2	5.6962	chr10q25	NM_005445
USP15	2.4623	3.6301	chr12q14	NM_006313
CDK6	2	3.3173	chr7q21-q22	NM_001259
WFDC1	0.0718	0.1191	chr16q24.3	NM_021197
DST	2.8284	2.9561	chr6p12-p11	NM_001723 /// NM_015548 /// NM_020388 /// NM_183380

Results

Drug-resistant phenotype and global gene expression analysis of Doc-resistant cell line. The Doc-resistant cell line was designated as BGC-823/DOC. There was no significant morphological difference between the two cell lines (Figure 1A). The growth curves and cell cycle analysis showed that the BGC-823/DOC cell line was resistant to Doc (Figures 1B-C). BGC-823/DOC also showed slight to moderate cross-resistance to vinblastine and paclitaxel, but not to cisplatin, oxaliplatin, 5-FU, gemcitabine or epirubicine (Supplementary Table IV).

Gene expression profiles of BGC-823 and BGC-823/DOC were examined using oligonucleotide microarrays. Among 14,500 genes analysed, expression levels of 328 and 155 genes increased and decreased, respectively, significantly in resistant cells compared to the parental cells. Gene functional analysis by gene ontology showed that functional classes of these differentially expressed genes included stress or cellular process, binding, metabolic process, biological regulation, catalytic activity and so on. Both up- and down-regulated genes elicited similar trends in functional classification (Figure 2).

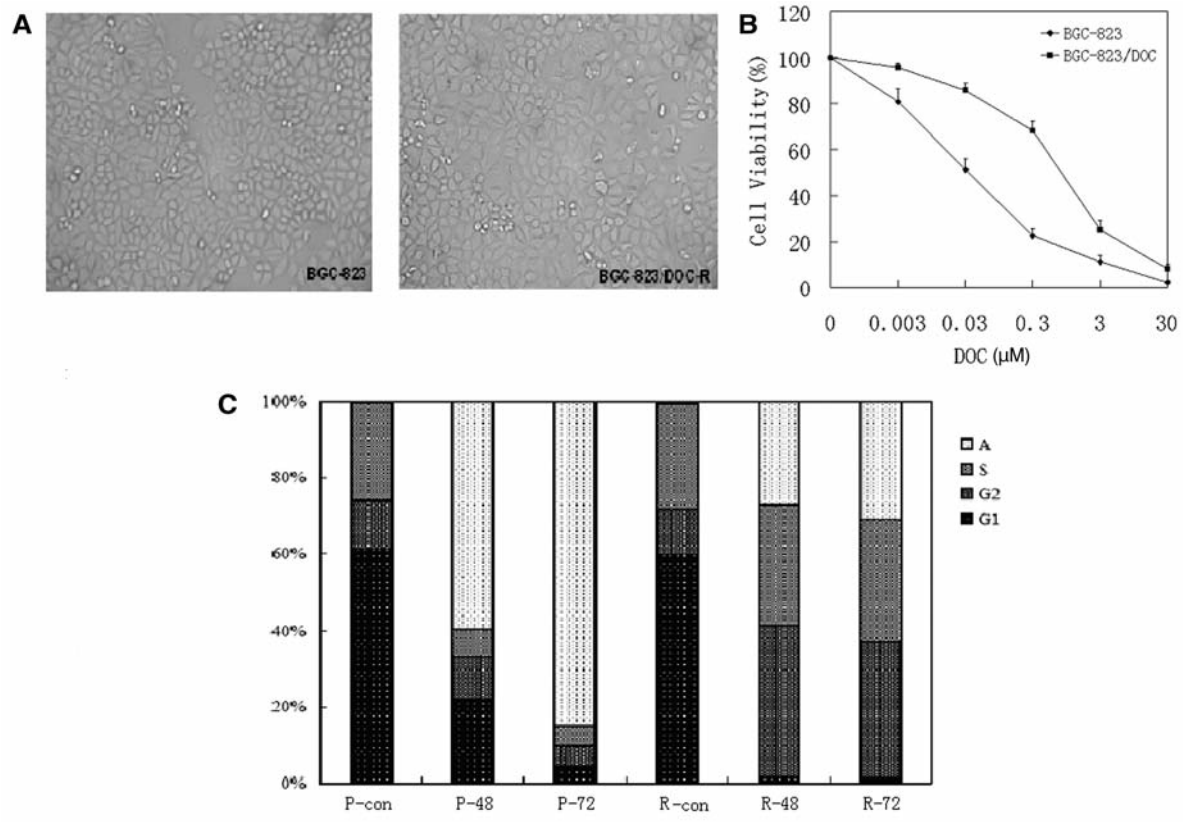

Figure 1. *Drug resistance phenotype of BGC-823/DOC. A: Morphology comparison between parental BGC-823 cells and docetaxel resistant BGC-823/DOC cells (100×). B: Cytotoxic effect of docetaxel on BGC-823 and BGC-823/DOC cells. C: Cell cycle arrest and apoptosis induced by docetaxel (0.5 μM) in BGC-823 and BGC-823/DOC cells. P, Parental cells; R, resistant cells; con, control without docetaxel treatment; 48, cells harvested after exposure to docetaxel for 48 h; 72, cells harvested after exposure to docetaxel for 72 h.*

Selection of candidate genes in cell lines. According to published studies and information from databases regarding gene functions, a panel of 11 genes was selected to validate the microarray results by quantitative RT-PCR. The expression levels of these genes shared the same trends when they were compared between parental and resistant cells using Q-PCR and microarray (Table I). Gene expression levels and docetaxel IC_{50}s were compared in 11 cancer cell lines of the digestion system (Supplementary Table V). Pearson correlation and Spearman correlation were used to compare mRNA expression levels and IC_{50}s of each cell line. *CDK6* (r=0.71, p=0.014), *USP15* (r=0.996, p<0.001), *CDH1* (r=–0.843, p=0.001) and *CXCR4* (r=0.609, p=0.047) were significantly correlated to docetaxel sensitivity.

Further selection using clinical gastric cancer specimens. The Doc sensitivity of gastric cancer specimens was assessed by HDRA. The inhibition rates ranged from 0 to 89.19%. The relative mRNA expression levels of the four candidate genes (*CDK6, USP15, CXCR4* and *CDH1*) were detected using Q-PCR in 25 primary gastric cancer specimens. Only

Table II. *Characteristics of gastric cancer specimens.*

	Resistant samples N(%)	Sensitive samples N(%)	*P*-value*
Age (years)			0.637
>60	7(36.8%)	2(33.3%)	
≤60	12(63.2%)	4(66.7%)	
Gender			0.198
Male	10(52.6%)	5(83.3%)	
Female	9(47.4%)	1(16.7%)	
Location			0.063
Distal	12(63.2%)	1(16.7%)	
Proximal	7(36.8%)	5(83.3%)	
Grade			0.637
G0-1	9(47.4%)	3(50.0%)	
G2-3	10(52.6%)	3(50.0%)	
Tumour (T)			0.219
1-2	5(26.3%)	0(0%)	
3-4	14(73.7%)	6(100%)	
Nodes (N)			0.070
0-1	8(42.1%)	0(0%)	
2-3	11(57.9%)	6(100%)	

*Fisher's exact test.

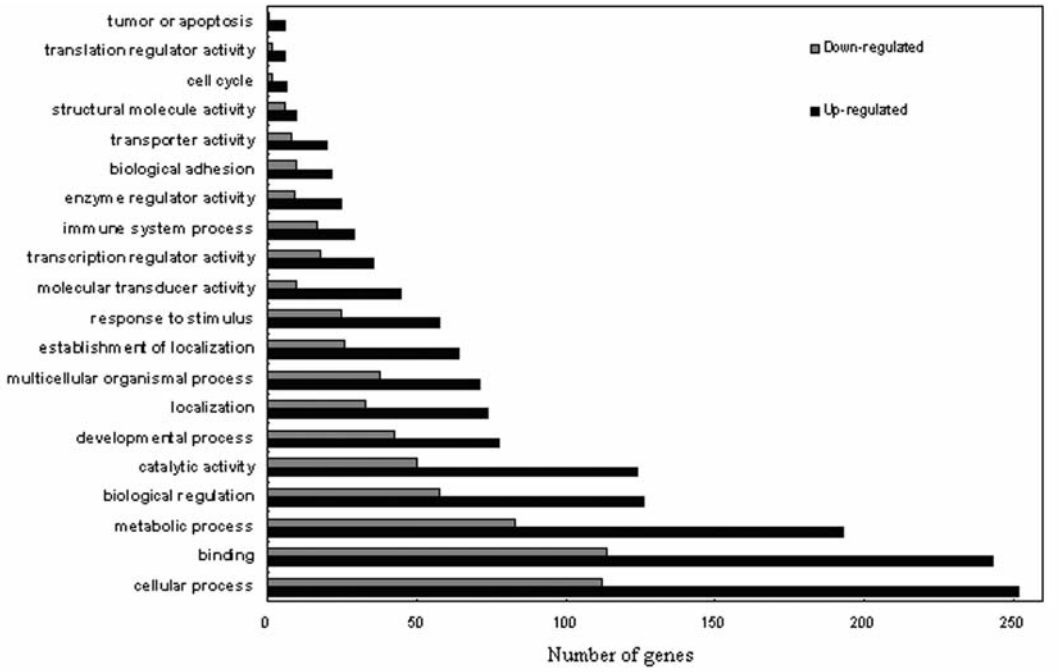

Figure 2. *Gene ontology analysis of genes changed by treatment of BGC-823/DOC cells with docetaxel. The expression of 328 and 155 genes which increased (up-regulated) and decreased (down-regulated) significantly, respectively, was analysed by gene ontology.*

CXCR4 was positively related to the inhibition rate of docetaxel in regression analysis (R^2=0.23, p=0.019. In advanced gastric cancer patients, a 19-24% response rate of docetaxel has been reported (15). Thus, the top 24% samples (6 samples) were defined as sensitive cases. No statistically significant correlations between docetaxel sensitivity and conventional clinical-pathological background factors were observed (Table II). However, the Doc-sensitive group had a significantly lower *CXCR4* expression than the resistant group (p=0.038, Figure 3).

Exploration of CXCR4 expression and Doc-treatment in vitro. The results described showed a paradoxical phenomenon. In the resistant cell line, the mRNA level of *CXCR4* was down-regulated, while the protein expression level was higher in the more resistant group of cell lines and tissue samples. To resolve this apparent paradox, the expression change of *CXCR4* was analysed in BGC-823 cells treated with Doc upon the induction of Doc resistance or acute exposure (Figure 4 A-B). A prominent *CXCR4* up-regulation occurred in the first 12 hours after DOC treatment. However, during the induction of resistant cells, the expression level of *CXCR4* was gradually down-regulated until it was finally lower than that of the parental cells. Thus, the lower level of *CXCR4* mRNA in resistant cells may have been due to the long-term drug treatment, with a certain compensatory function occurring.

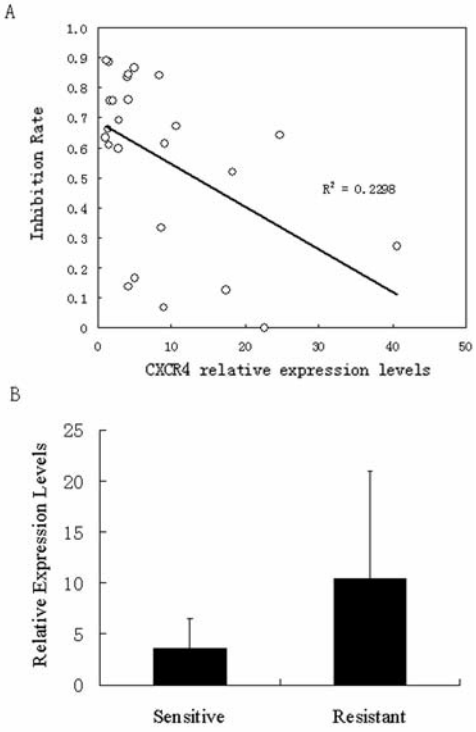

Figure 3. *CXCR4 mRNA is related to docetaxel sensitivity in gastric cancer. A: Correlation between CXCR4 mRNA expression levels and inhibition rate of docetaxel. B: Mean CXCR4 expression levels of the sensitive and resistant groups (mean CXCR4 expression ± standard deviation).*

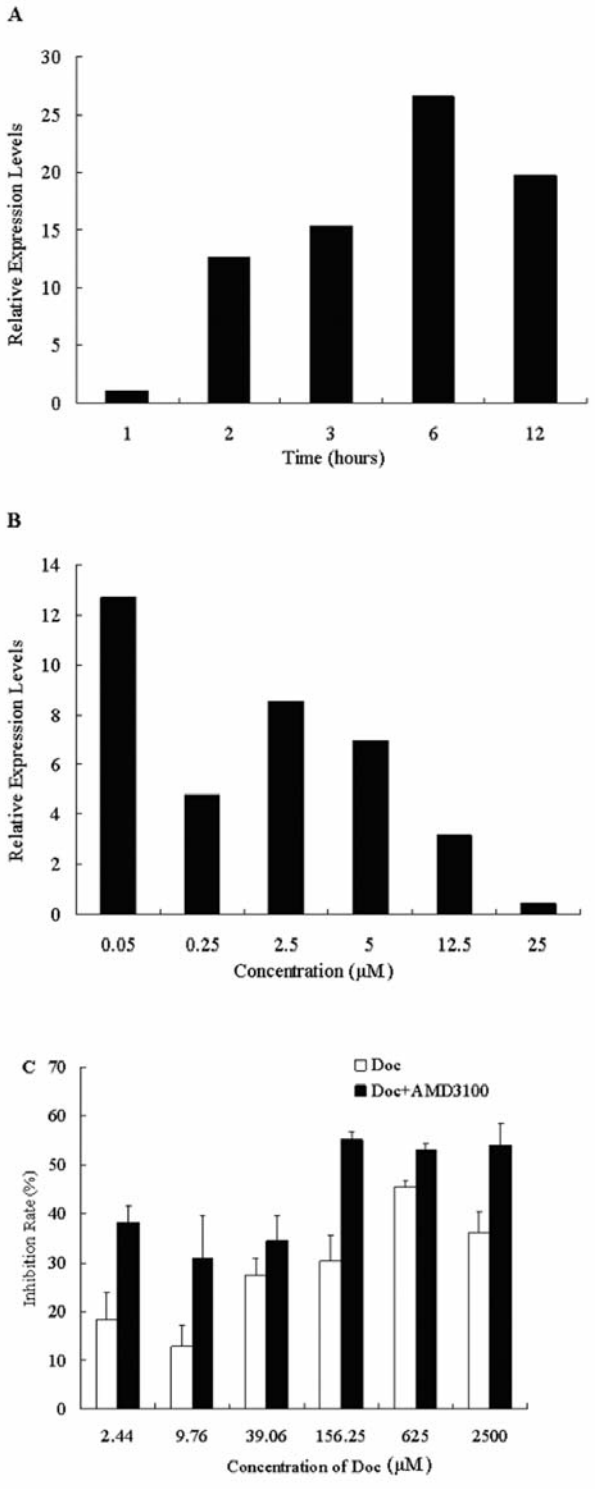

Figure 4. *The possible role of CXCR4 in docetaxel sensitivity. A: CXCR4 mRNA expression levels were up-regulated when BGC-823 cells were treated with docetaxel at the IC_{50} (10 nM) for 2 to 12 h; B: CXCR4 mRNA expression levels decreased during the induction of resistance for BGC-823 cells; C: AMD3100 (200 ng/ml) enhanced cytotoxicity of docetaxel on MKN-45 cells using an MTT assay (mean inhibition rates ± standard deviation).*

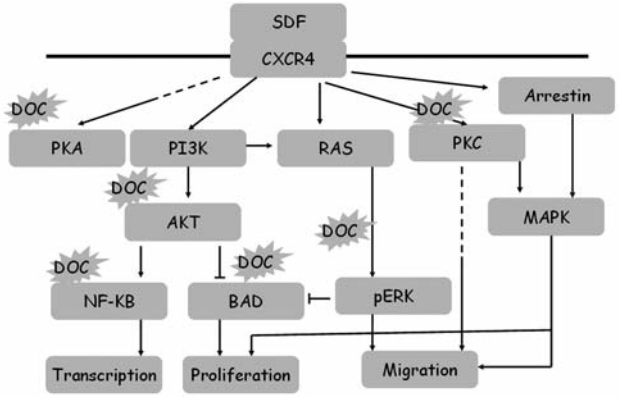

Figure 5. *CXCR4 signaling pathway and possible role in docetaxel (DOC) sensitivity. The signal pathway is plotted according to Wong et al. (28). Blocking the signaling pathway with antagonists or siRNA against PKA (29), AKT (30), NF-κB (31) and ERK(32) can sensitise cancer cells to Doc. The tvarious isoforms of PKC can also affect the Doc sensitivity. Medicines which can down-regulate NF-κB and ERK or phosphorylate BAD (35) can also change chemosensitivity to Doc. Thus, the signal pathway activated by CXCR4 was related closely to the sensitivity to Doc.*

Next, the *CXCR4* antagonist AMD3100 was used to block the CXCR4 signal pathway in MKN-45 cells, which have the highest CXCR4 expression among the gastric cancer cells used in this study. When treated with AMD3100 at a concentration of 200 ng/ml, MKN-45 cells grow without significant cell proliferation inhibition ($p>0.05$, supplementary Figure 1). However, AMD3100 enhanced the cytotoxity of Doc when MKN-45 cells were treated with Doc and AMD3100, simultaneously (Figure 4C).

Discussion

Measuring the mRNA levels of a panel of genes using real-time PCR is potentially useful in predicting drug efficiency for clinical applications (7). In the current study, *CXCR4* mRNA levels were found to be correlated with Doc sensitivity of cancer cell lines and cancer tissues. This finding indicates that CXCR4 may be a potential biomarker for Doc chemosensitivity.

CXCR4 is crucial in the homing of haematopoietic cells and the metastasis of solid tumours such as gastric cancer (16-18). Activation of CXCR4 leads to enhancement of cell motility, which is characterised by the increased formation of filopodia, uropods, and neurite-like projections (19). CXCR4 is involved in the regulation of the cell skeleton and also partly in the dynamics of microtubules, where tubulin-stabilising reagent acts as an antagonist. In this regard, the activation of CXCR4 signaling may result in Doc resistance (20). Several studies have shown that CXCR4 is related to cancer stem cells (CSCs)

Table III. *Primer Information*.

Gene symbol	Primers
SMC3	F: 5'-CGAGCCCTGGAATATACCATTTACA-3'
	R: 5'-CTCTTGCATCCTGCTGAGCATC-3'
DST	F: 5'-AATCTCCAGTGAAACGCCGAAG-3'
	R: 5'-TCATGAGAGTGACCAGGGCAGTA-3'
USP15	F: 5'-GAATGGCCCAAATGGCATACA-3'
	R: 5'-ATCCTGGCTGGATTCATCATCTG-3'
CDK6	F: 5'-AGAAGAAGACTGGCCTAGAG-3'
	R: 5'-TGGAAGTATGGGTGAGACAGG-3'
APC	F: 5'-TGTCCCTCCGTTCTTATGGAA -3'
	R: 5'-TCTTGGAAATGAACCCATAGGAA-3'
CCL5	F: 5'-TACCATGAAGGTCTCCGC-3'
	R: 5'-GACAAAGACGACTGCTGG-3'
CDH1	F: 5'-TGAAGGTGACAGAGCCTCTGGAT-3'
	R: 5'-TGGGTGAATTCGGGCTTGTT-3'
CXCR4	F: 5'-CCTATGCAAGGCAGTCCATGT-3'
	R: 5'-GGTAGCGGTCCAGACTGATGA-3'
DCN	F: 5'-TCAATGGACTGAACCAGATGA-3'
	R: 5'-CCTTGAGGAATGCTGGTGAT-3'
PIK3CA	F: 5'-CCTGATCTTCCTCGTGCTGCTC-3'
	R: 5'-ATGCCAATGGACAGTGTTCCTCTT-3'
ACTB	5'-TTCCTGGGCATGGAGTC-3'
	5'-CAGGTCTTTGCGGATGTC-3'

Table IV. *Cross-resistance data*.

Drug	IC_{50} (μM)		Resistance index
	BGC-823	BGC-823/DOC	
Paclitaxel	0.05±0.01	1.58±0.08	31.6
Cisplatin	7.19±0.29	7.01±0.32	0.98
Oxaliplatin	12.13±1.62	12.37±1.47	1.02
5-Fluorouracil	35.06±1.03	39.62±3.25	1.13
Gemcitabine	1.36±0.24	0.85±0.07	0.63
Epirubicine	0.16±0.01	0.21±0.03	1.34
Vincristine	0.06±0.01	0.37±0.07	5.57

which are thought to be the root of drug resistance (18, 21). The CXCR4 expression in cancer tissue may partially reflect the number of CSCs, and thus indicate chemotherapeutic resistance as well as poor prognosis (22, 23). CXCR4 inhibitor has also been found to be able to sensitise malignant cells to chemotherapeutic reagents *in vivo* by disrupting adhesion and homing to bone marrow niches (24, 25).

There are several studies showing that CXCR4 is involved in cell proliferation in specific cancer types, usually *in vivo*, and that CXCR4 antagonists have an anti-proliferative potential (26). High proliferation potential as well as apoptosis always results in chemoresistance (27). The CXCR4 signaling pathway is related to several important signal transduction pathways such as PI3K/AKT, Ras/Raf/ERK (28, Figure 5). Small molecular inhibitors of ERK, NF-κB, and AKT, as well as siRNAs targeted to these genes, can sensitise cancer cells to Doc (29-32). Most pathways activated by CXCR4 play important roles in the resistance to Doc (Figure 5) (33, 34), hence up-regulation of CXCR4 may also result in resistance to Doc.

In the present study, the CXCR4 pathway was blocked by AMD3100 and Doc sensitivity was enhanced in MKN-45 cells. However, further studies are still necessary, focusing on the underlying mechanisms such as the up- or down-regulation of *CXCR4* expression. The results of the present study were based on *in vitro* experiments which cannot replace an *in vivo* study. Although the published literature supports the present finding that CXCR4 can regulate sensitivity to Doc, the possibility that CXCR4 is only a predictive biomarker without important function still remains and further studies are necessary.

In conclusion, a specific mRNA expression profile of BGC-823/DOC cells was obtained against BGC-823 cells. After two steps of selection using cell lines and tissue samples, CXCR4 was found to be a potential biomarker related to sensitivity to

Table V. *Gene expression level and IC_{50} of 11 cancer cell lines*.

	CaEs	KATOIII	AGS	MKN-45	SGC-7901	LS174T	LoVo	SMMC-7721	HeG2	QGY-7701	Panc-1
IC_{50}	32.4625	7.2	13.7365	486.07	47.09	11.1967	16360.96	2588.5	173.9867	456.1875	68.9567
APC	2.7702	0.3403	10.5927	3.5186	1.5529	0.8207	0.4553	47.0134	0.2031	2.3214	2.4708
CCL5	0	1.257	0	22.5491	16.4498	0	0	0	0.1077	0.4914	0.1599
SMC3	1.7901	0.8675	2.4453	4.9075	1.4692	0.3737	0.6552	0.8919	0.0887	2.0209	1.2269
DST	1.5	0.2491	0	6.7272	10.6664	0	0	1.1173	0.0854	0.4585	4.1267
PIK3A	0.8675	2.1886	0.1241	0.4649	0.1914	6.3203	19.1597	100.7754	0.1259	5.8159	7.1354
USP15	1.5	0.2973	0.7928	1.3149	0.727	1.8213	28.7402	6.5206	0.0398	0.8888	0.6484
CDK6	0.5987	0.1492	0.2973	0.6242	1.5422	2.0279	2.9079	1.7901	0.0788	0.0094	0.3673
CDH1	0.0854	1.0718	0.1303	0.0305	0.047	7.9173	0	0	0.7423	0.0334	0.483
CXCR4	0.1875	1.1096	0.2238	2.2038	0.9626	0.2892	3.9041	8.1117	0.0485	3.7711	0.8409
DCN	0.4248	0.0005	0	0.1088	0.0081	0	0	0	0.125	1.6415	1.1769
WFDC1	18.2522	0	10.6974	4.4774	4.6913	0	0	0	4.6751	0	0.2793

Doc. A new potential Doc-sensitivity predictive biomarker was proposed, which may contribute to increased predictive accuracy when combined with other predictors. Further studies focusing on *CXCR4* mRNA expression levels and Doc treatment are necessary to confirm their relationship.

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