

## Original Article

# AKT/ERK activation is associated with gastric cancer cell resistance to paclitaxel

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**Abstract:** Paclitaxel (PTX) has shown encouraging activity in the treatment of advanced gastric cancer (GC). However, the fact that more than half of GC patients respond poorly to PTX-based chemotherapies demonstrates the urgent need for biomarkers of PTX sensitivity in GC patients. In the present work, three GC cell lines (BGC-823, HGC-27 and NCI-N87) with different sensitivities to PTX were subjected to DNA microarray analysis. The significantly differentially expressed genes and microRNAs (miRs) were identified and pathway signatures for PTX sensitivity were proposed. Ingenuity Pathway Analysis results showed that the differentially expressed genes were mainly enriched in the ErbB signaling pathway and other pathways. Additionally, the AKT/ERK signaling pathway, which is the pathway downstream of ErbB, was predicted to be active in PTX-resistant GC cell lines. ErbB3 overexpression and AKT/ERK activation in PTX-resistant cell lines were validated, respectively, by quantitative PCR and immunoblotting. Furthermore, 10 miRs were dramatically differently expressed in the three GC cell lines, and a miR-gene network was constructed from these data. Our work uncovered a reliable signature for PTX sensitivity in GC and potential therapeutic targets for GC treatments.

**Keywords:** Gastric cancer, paclitaxel, DNA microarray, ErbB signaling, AKT signaling, ERK signaling, microRNA

## Introduction

Gastric cancer (GC) is one of the most common human cancers and ranks second in global cancer-related mortality. The clinical outcome of patients with advanced gastric cancer (AGC) is markedly dependent on their response to chemotherapy. Paclitaxel (PTX), one of the most promising cytotoxic agents in clinical use, has shown encouraging activity in various studies as a single agent or as part of combination regimens in the treatment of advanced gastric cancer. PTX in combination with capecitabine is the first-line chemotherapy for AGC in China, although the overall response rate (ORR) is below 50% [1]. In phase 2 trials, PTX plus 5'-deoxy-5-fluorouridine (5'-DFUR) or S-1 generates an ORR of 40.5-46.3% for unresectable or recurrent GC [2, 3]. When used alone as a second-line therapy for docetaxel-refractory AGC patients, PTX generates an ORR of 12.5-14.2% [4, 5]. Furthermore, PTX also has antitumor activity against local AGC in adjuvant or neoadjuvant chemotherapy [6-8].

Collectively, more than half of AGC patients respond poorly to PTX-based chemotherapies, demonstrating the need for uncovering biomarkers for PTX in GC cells.

Previous studies have proposed that dozens of genes and several microRNAs (miRs) are associated with the sensitivity of GC cells to PTX. The most exceptional associated gene is Class III beta-tubulin (TUBB3) [9-12]. PI3K/AKT/mTOR signaling [13, 14], NF- $\kappa$ B signaling [15, 16], SRC [17], FGFR2 [18], VEGFR2 [19], HBEGF [20] and CHK2 [21] are also all related to PTX sensitivity. Moreover, miR-27a [22], miR-23a [23] and miR-34c-5p [24] are suggested to regulate the sensitivity of cancer cells to PTX. However, a systematic evaluation of these biomarkers in GC patients remains challenging, and it is of great interest to investigate putative biomarkers in vitro in GC cell lines.

In the present work, three GC cell lines with different sensitivities to PTX were subjected to DNA microarray analysis. The differently expressed

## AKT/ERK is associated with GC resistance to paclitaxel

**Table 1.** Primers used for qPCR validation

GENE	Forward	Reverse
Actb	CACCATGTACCCTGGCATT	GTACTTGCCTCAGGAGGAG
ICAM1	CCTCCCACCCACATACATTT	GTCCAGACATGACCGCTGAGT
KSR1	GCAAGCATTGCAGGTTGAAG	CCTCCGAAGCCGAGTTAGTG
MMP2	CGGCGGTCACAGCTACTTC	TTCACGCTCTTCAGACTTTGGTT
IL12B	ACCATCCAAGTCAAAGAGTTTGG	AGGAGCGAATGGCTTAGAACCT
MYC	GGCGAACACACAACGTCTTG	TGGTCACGCAGGGCAAA
ADCY7	CACACTACTGCCCTTCAGCA	AAGCCTCCCATCAAAGAACC
TAF2	AGAGCCCGCCAGAATGAAC	GCAGACGACCTGATGGGTTAAT
TGFB1	AATTGAGGGCTTTCGCCTTAG	TGAACCCGTTGATGCCACTT
PRKCH	CTGGACCCCTATCTGACGGT	TGTACGTGGGTTTGTGGTCT
ERBB3	GTCATGAGGGCGAACGAC	AGAGTCCAGGACACTGAC

ssed genes and miRs were identified and pathway signatures for PTX sensitivity were proposed.

### Materials and methods

#### Cell culture

BGC-823, HGC-27 and NCI-N87 cell lines were purchased from ATCC and maintained in DMEM or RPMI 1640 medium supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Life Technologies). Cells in the exponential growth phase were used for all experiments.

#### MTS assay of cell line viability

Cells ( $4 \times 10^3$  per well) were cultured in 100 µl DMEM or RPMI 1640 medium containing serum in a 96-well plate. After 24 h, the cells were treated with PTX (0.001, 0.0032, 0.01, 0.032, 0.10, 0.32, or 1.00 µmol/L) for 72 h. Each treatment was assayed in triplicate in the same experiment. Then, 20 µl of MTS (CellTiter 96 Aqueous One Solution Reagent; Promega) was added to each well for 2 h at 37°C. After incubation, the absorbance was read at 490 nm according to the manufacturer's protocol. The IC50 calculation was performed with GraphPad Prism 5.0 software.

#### Microarray analysis

Three cell lines ( $8 \times 10^4$  per well) were grown in 2 ml of DMEM medium containing serum per well in a 6-well plate in duplicate. All of the samples were homogenized in 1 ml Trizol (Invitrogen, Life Technologies), and total RNAs were ex-

tracted according to the manufacturer's instructions.

Total RNA (500 ng) was used to synthesize double-stranded cDNA, which was transcribed in vitro to cRNA. Purified cRNA (10 µg) was used to synthesize 2nd-cycle cDNA, which was then hydrolyzed with RNase H and purified. The above steps were performed with the Ambion WT Expression Kit. Second-cycle cDNA (5.5 µg) was fragmented, and the single-stranded cDNA was labeled with the GeneChip2 WT Terminal Labeling

Kit and Controls Kit (Affymetrix, PN 702880). Approximately 700 ng of fragmented and labeled single-stranded cDNA was hybridized to an Affymetrix GeneChip Human Gene 1.0 ST array, which was washed and stained with the GeneChip2 Hybridization, Wash and Stain kit (Affymetrix).

Microarray data analysis was performed using the Significance Analysis of Microarrays (SAM) method, as previously described before [25]. Functional annotation of the differentially expression genes was performed with Ingenuity Pathway Analysis (IPA) online software.

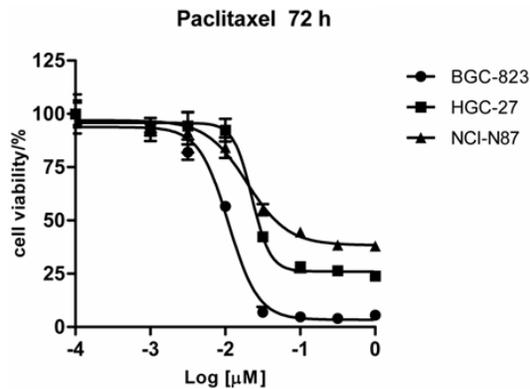
#### Quantitative real-time PCR (qPCR)

Total RNA isolated as above was synthesized into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR using a mixture of oligo-dT and random primers (9-mer). The primers used for qPCR validation are listed in **Table 1**. Real-time qPCR was performed with CFX-96 (Bio-lab), using hActb as the endogenous control. Gene expression was calculated relative to expression of the hActb endogenous control and was adjusted relative to expression in BGC-823 cells.

#### Protein isolation and western blotting

Cell pellets were resuspended in 1× SDS loading buffer (1 mmol/L  $\text{Na}_3\text{VO}_4$ , 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 µg each lane) were run on SDS-PAGE. Immunoblotting with antibodies specific for GAPDH (Abmart, 080922), AKT (Santa Cruz, sc-8312), p-AKT (Santa Cruz, SC-

## AKT/ERK is associated with GC resistance to paclitaxel



**Figure 1.** Three GC cell lines exhibited different sensitivity to PTX. BGC-823, HGC-27 and NCI-N87 cells were treated with 7 different concentrations of PTX or were untreated for 72 h. Cell viability was determined using an MTS assay, and the survival curve was plotted.

7985-R, pS473), ERK (Abclonal, A0228) and p-ERK (Cell Signaling, #9106S, pT202/204) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) antibodies and visualized using a chemiluminescence detection system (Millipore, WBKLS0500).

### *miR target prediction and miRNA target correlation*

miR target prediction was performed with miRWalk online software. Comparative analysis was performed with 5 prediction programs: miRanda, miRDB, miRWalk, RNA22 and TargetScan. miR target prediction was performed on 10 miRs. Genes predicted by at least 3 programs were selected as putative downstream targets of the candidate miR. The predicted target genes of miRs with increased expression were compared to genes with decreased expression as assessed by microarray; accordingly, the predicted target genes for miRs with decreased expression were compared to genes with increased expression. The overlapping genes were used to construct miR-gene networks using Cytoscape 2.8 software.

## Results

### *Three GC cell lines exhibit different sensitivity to PTX*

The GC cell lines BGC-823, HGC-27 and NCI-N87 were treated with 7 concentrations of PTX (0.001, 0.0032, 0.01, 0.032, 0.10, 0.32, or

1.00 µmol/L) or were untreated for 72 h. Cell viability was detected using the MTS assay, and the relative IC<sub>50</sub> was calculated (**Figure 1**). The IC<sub>50</sub> doses to PTX for BGC-823, HGC-27 and NCI-N87 at 72 h were 10, 48 and 124 nmol/L, respectively. The BGC-823 cell line is relatively sensitive to PTX, whereas the HGC-27 cell line is moderately sensitive to PTX. The bottom of the survival curve for the NCI-N87 cell line was approximately 40%, indicating that the NCI-N87 cell line is resistant to PTX.

### *DNA microarray analysis: mRNA and miR expression profiles*

The basal gene expression of the 3 GC cell lines was investigated by DNA microarray, and the differences in expression pattern were analyzed between these GC cell lines. The expression of 68 genes (e.g. MAP2K1, NRG1) increased, and the expression of 55 genes (e.g. BCL2L11, ADCY7) decreased by more than 50% in BGC-823 compared to HGC-27 and in HGC-27 compared to NCI-N87.

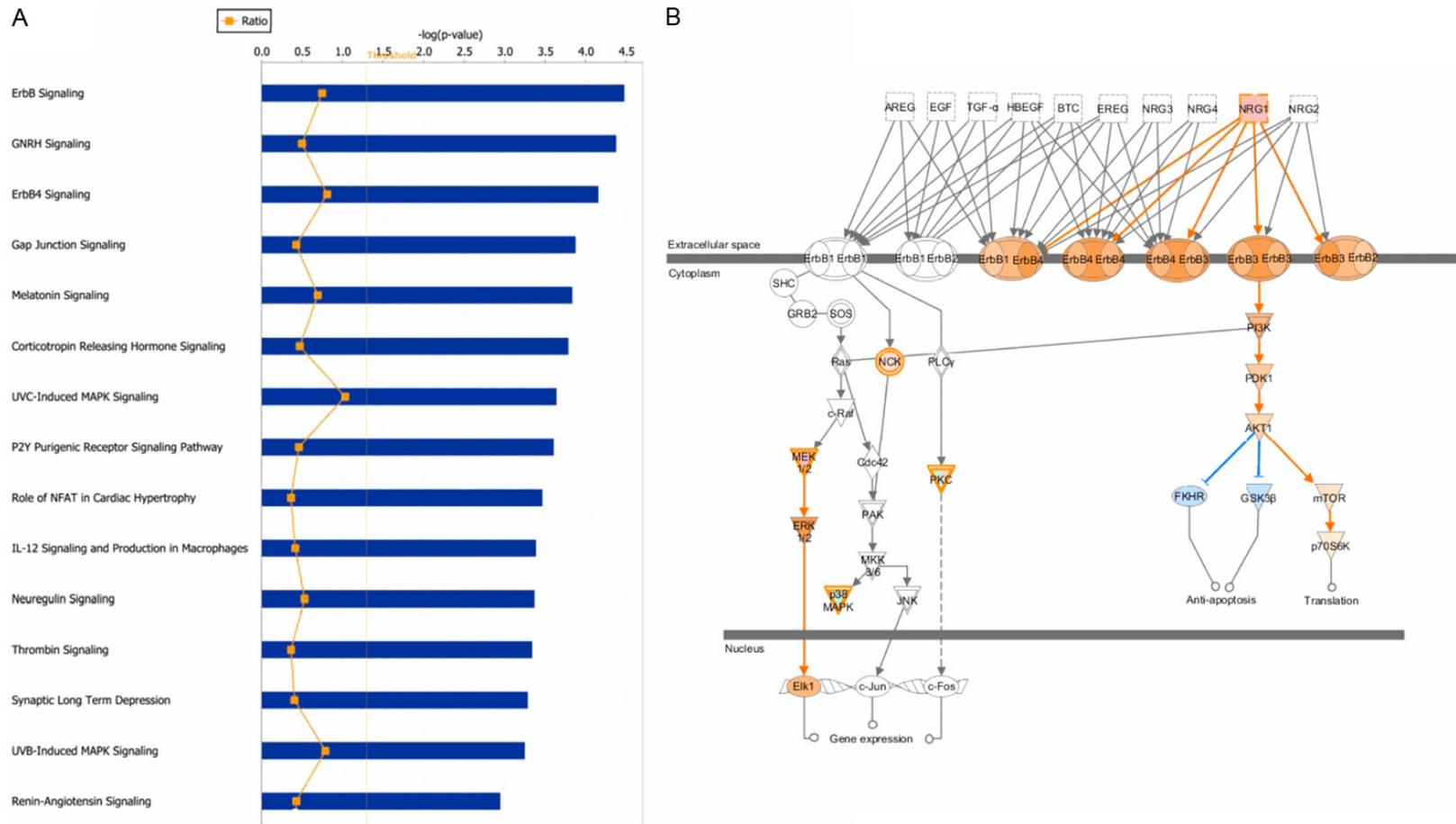
The 123 identified genes (68 upregulated genes and 55 downregulated genes) were examined using Ingenuity Pathway Analysis (IPA). The IPA results showed that the differently expressed genes were enriched for ErbB signaling, GNRH signaling, ErbB4 signaling and some additional pathways (**Figure 2A**). ErbB signaling was predicted to be activated in the PTX-resistant NCI-N87 cell line (**Figure 2B**), mainly based on the upregulation of MAP2K1 (MEK1) and NRG1; therefore downstream ERK and AKT/mTOR/p70S6K signaling was predicted to be activated.

Concurrently, the miR expression between the 3 GC cell lines was investigated using miR expression chips. The expression of 10 miRs was more than 50% higher in BGC-823 compared to HGC-27 and in HGC-27 compared to NCI-N87. Among these miRs, 7 (miR-224, miR-424-3p, miR-130a, miR-224-star, miR-452, miR-181a-2-star, and miR-193b-5p) were downregulated in the PTX-resistant GC cell line, whereas the other 3 miRs (miR-3127-5p, miR-1287, and miR-4713-5p) were upregulated in the PTX-resistant GC cell line compared to the PTX-sensitive GC cell line (**Table 2**).

### *qPCR validation of DNA microarray data*

To further analyze the differences in gene expression, qPCR was performed on 10 genes

## AKT/ERK is associated with GC resistance to paclitaxel



**Figure 2.** Ingenuity Pathway Analysis. A: The most significant canonical pathways in which differently expressed genes were enriched. The 123 genes identified as differently expressed (expression difference >1.5-fold) were examined using Ingenuity Pathway analysis (IPA) software, and the most significant canonical pathways are shown. B: The predicted increase in ErbB signaling in the PTX-resistant GC cell line is shown. The prediction was based on the expression of associated genes assessed using the DNA microarray data. The orange circle and arrow represent “induce”, and the blue circle and arrow represent “inhibit”.

**Table 2.** The top 10 expression-altered miRs between 3 GC cell lines

miR	HGC27 vs BGC823		NCI-N87 vs HGC27	
	Fold change	P value	Fold change	P value
miR-224	0.16	0.001	0.03	0.001
miR-424-star	0.40	0.03	0.01	0.001
miR-130a	0.48	0.04	0.02	0.001
miR-224-star	0.18	0.005	0.04	0.002
miR-452	0.18	0.02	0.07	0.002
miR-181a-2-star	0.21	0.01	0.28	0.02
miR-193b-star	0.20	0.02	0.30	0.03
miR-3127-5p	1.65	0.03	3.74	0.01
miR-1287	2.77	0.01	2.62	0.03
miR-4713-5p	2.36	0.03	1.67	0.24

from BGC-823 and NCI-N87 to validate the DNA microarray data. The expression fold change in NCI-N87 compared to BGC-823 was log<sub>2</sub> transformed and plotted (**Figure 3**). The fold change varied between qPCR and microarray data; however, the trend in the expression of most of the genes between the microarray dataset and the qPCR dataset was consistent except for 3 genes. MMP2, IL12B and MYC were downregulated in the PTX-resistant NCI-N87 cell line in microarray data, whereas the qPCR data showed that these 3 genes were upregulated in NCI-N87 cells. These results suggested that although DNA microarray data can be used to systematically screen for candidate genes, the expression changes to observed genes requires further validation when using microarray data for functional annotation.

*AKT/ERK signaling was active in the PTX-resistant GC cell line*

Because the IPA results indicated that AKT/ERK signaling was active in the PTX-resistant GC cell line, the status of AKT/ERK signaling was examined by immunoblotting (**Figure 4**). BGC-823, the PTX-sensitive cell line, has decreased levels of phosphorylated AKT and ERK signaling. HGC-27, the PTX-moderately sensitive cell line, has higher levels of phosphorylated AKT but ERK signaling is not active. NCI-N87, the PTX-resistant cell line, has higher levels of phosphorylated AKT and active ERK signaling. Overall, AKT/ERK signaling was active in the PTX-resistant GC cell line, which in cancer cells might cause resistance to PTX.

*miR-gene network construction*

miRs regulate gene expression transcriptionally or post-transcriptionally [26]. A large number of miRs and mRNAs were expressed differently in the GC cell lines. We speculated that the altered expression of some of the genes in the GC cell lines was caused by changes in miR expression. To address this possibility, a network between the differentially expressed miRs and mRNAs in the GC cell lines was constructed. The 10 significantly differentially expressed miRs were selected, and their downstream targets were predicted using the online software

miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>). The predicted miR targets were compared to the differently expressed genes. Overlapping genes might be putative targets of the selected miRs. Then, a network was constructed using Cytoscape 2.8. The results showed that the network contains 6 miRs and 33 genes (**Figure 5**).

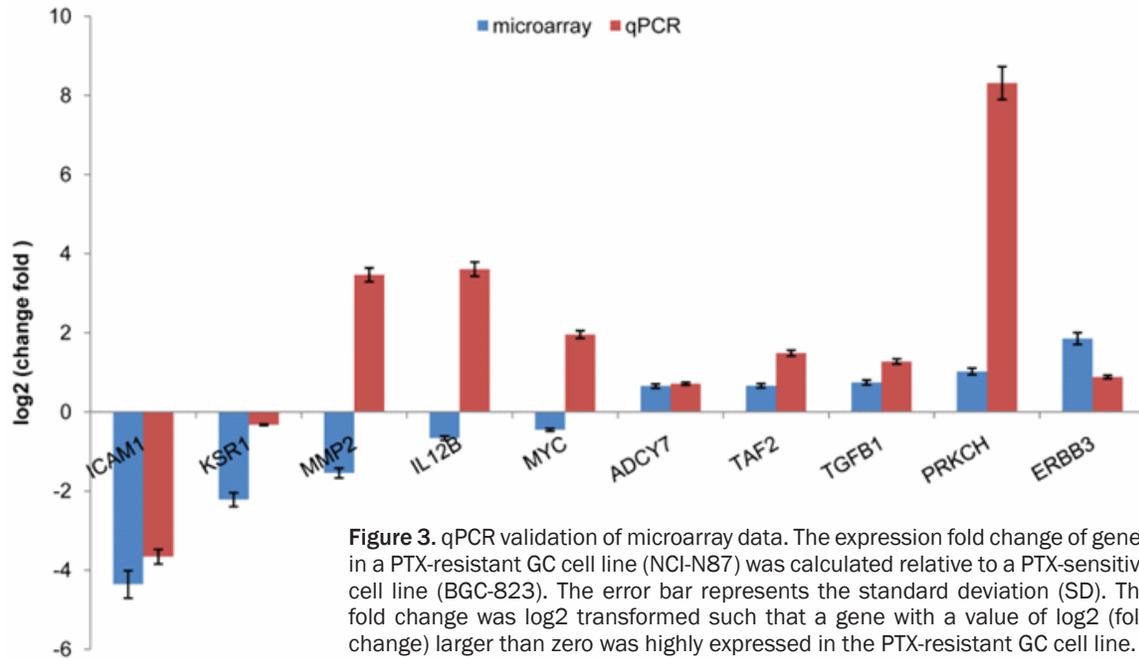
**Discussion**

PTX has been widely used in the clinical treatment of various cancers, including gastric cancer. However, more than half of GC patients do not respond to PTX-based chemotherapies. This demonstrates the urgent need for biomarkers of PTX sensitivity in GC patients.

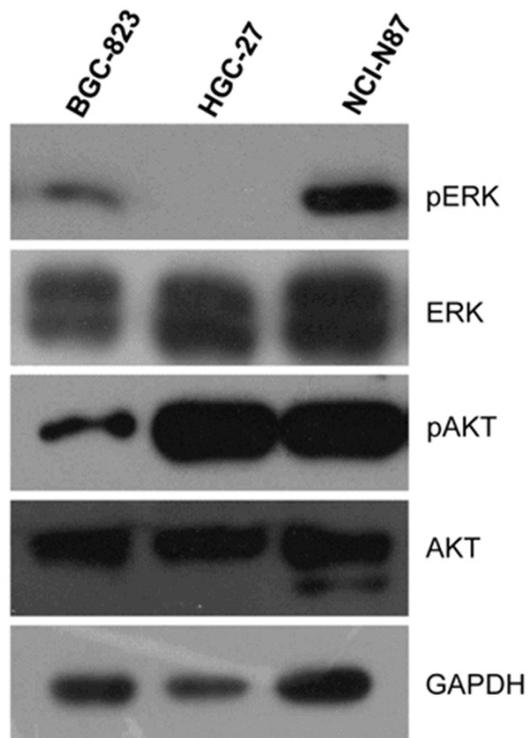
In our work, three GC cell lines, BGC-823, HGC-27 and NCI-N87, display significantly different sensitivities to PTX. In theory, the different sensitivity to PTX should be caused by the different basal expression of some genes. Hence, DNA microarray analysis performed on these GC cell line, and differently expressed genes and miRs were identified based on sensitivity to PTX. The expression of 68 genes increased, and the expression of 55 genes decreased more than 50% in BGC-823 compared to HGC-27 and in HGC-27 compared to NCI-N87.

Of the 123 identified genes, IPA found that many were involved in ErbB signaling, GNRH signaling and other signaling pathways. The HER2 protein (p185, HER2/neu, ErbB-2) is a 185-kDa transmembrane tyrosine kinase (TK) receptor and a member of the epidermal growth

## AKT/ERK is associated with GC resistance to paclitaxel



**Figure 3.** qPCR validation of microarray data. The expression fold change of genes in a PTX-resistant GC cell line (NCI-N87) was calculated relative to a PTX-sensitive cell line (BGC-823). The error bar represents the standard deviation (SD). The fold change was log<sub>2</sub> transformed such that a gene with a value of log<sub>2</sub> (fold change) larger than zero was highly expressed in the PTX-resistant GC cell line.

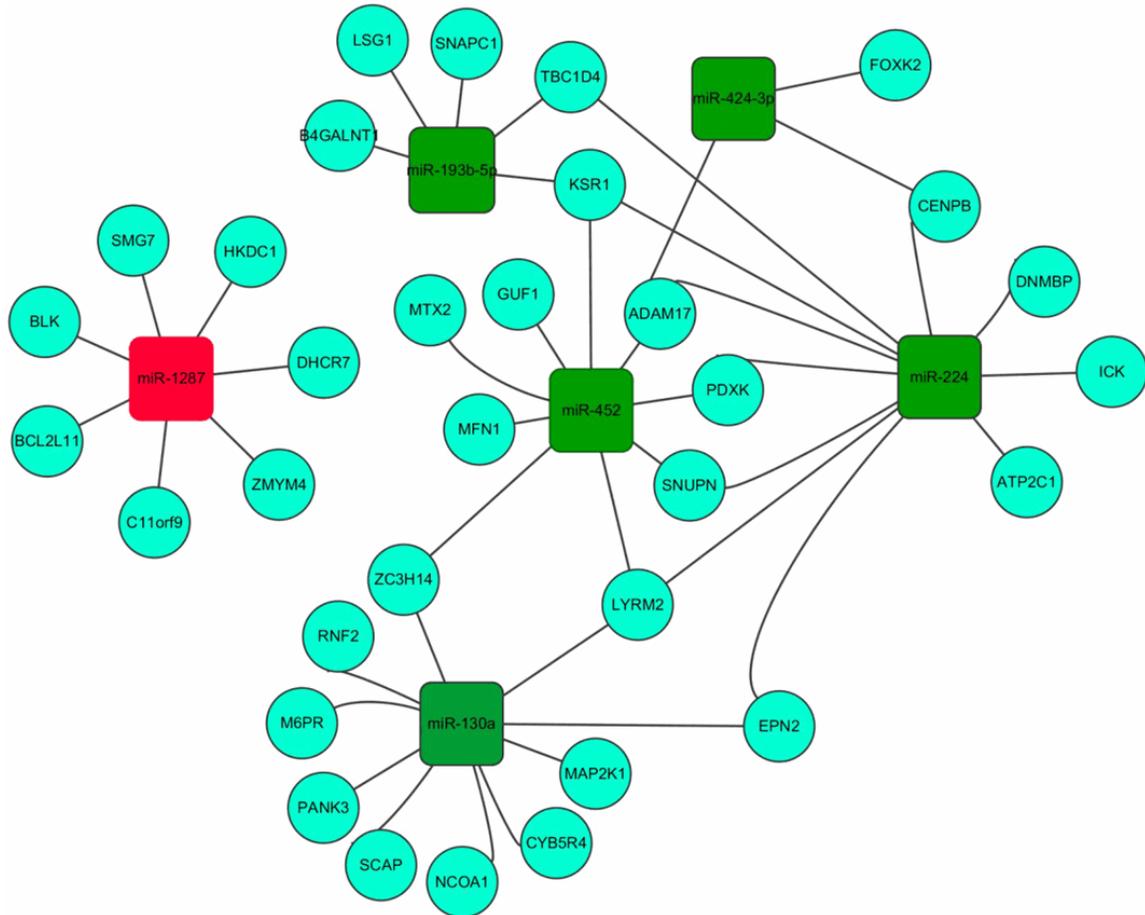


**Figure 4.** Immunoblotting of AKT/ERK for three GC cell lines. Total proteins from BGC-823, HGC-27 and NCI-N87 cells were subjected to SDS-PAGE and blotted onto a PVDF membrane. The protein expression levels of AKT, p-AKT, ERK and p-ERK were examined.

factor receptors (EGFR) family. Recent studies propose a role for HER2 in the development of

numerous types of human cancer. HER2 overexpression and/or amplification have been detected in various cancers, including gastric cancer. HER2 overexpression/amplification is observed in 5-25% of GC cases [27] and solidly correlates to the poor outcomes and a more aggressive disease in GC [28], suggesting that this gene might serve as a new prognostic factor and novel therapeutic target. In the three GC cell lines used in this work, NCI-N87 is a HER2-amplified GC cell line [29], whereas HGC-27 harbors mutations in PIK3CA and TP53, and an inactivated PTEN gene [30, 31]. BGC-823 has mutations in TP53 [31]. Previous studies suggest that EGFR/ErbB3 mutation or overexpression causes cancer cell resistance to PTX [32-34]. It is known that HER2 does not bind to any known ligand but prefers to heterodimerize with other HER family members, mainly EGFR and ErbB3. In our data, HER2-amplified NCI-N87 shows high levels of ErbB3 expression, which was validated by qPCR. It has been reported that transient induction of ErbB3 expression activates AKT and inhibits paclitaxel-induced apoptosis in ErbB2-overexpressing breast cancer cells [34]. Therefore, it seems likely that activation of the ErbB signaling pathway rather than a single ErbB protein causes cancer cell resistance to chemotherapy. Furthermore, our work demonstrated that AKT and ERK signaling, which is downstream of the ErbB pathway, were active in the PTX-resistant

## AKT/ERK is associated with GC resistance to paclitaxel



**Figure 5.** MiR-gene network. The 10 miRNAs with the most significantly changes in gene expression were examined with miRWalk online software to predict the downstream putative target genes. The identified targets were compared to the genes with altered expression (68 with increased expression and 55 with decreased expression in the PTX-resistant cell line) determined by microarray. The overlapping genes were considered potential downstream targets and used to construct the miR-gene network using Cytoscape 2.8 software. The red square represents the miRNAs with increased expression, and the green squares represent the miRNAs with decreased expression. The circle represents the gene; the gray line represents the regulation of the gene by miRNAs.

NCI-N87 cell line, whereas the cell line moderately sensitive to PTX. HGC-27, expresses active AKT but not ERK signaling, possibly because of the synergistic effect of PIK3CA mutation and the loss of PTEN in this cell line. Several studies suggest that AKT and/or ERK (AKT/ERK) inhibition contributes to overcome PTX resistance in various types of cancer [35-39]. Furthermore, NF- $\kappa$ B signaling and p38-MAPK signaling have been reported to be involved in resistance to PTX in some cases [35, 39, 40]. In general, AKT/ERK signaling activation results in the resistance to PTX, which warrants further validation in additional GC cell lines and tissues. Moreover, the activation of AKT/ERK may cause cancer cells to develop multidrug resistance, which has

been suggested by previous studies [32, 41]. This is another subject that deserves further investigation.

Collectively, our work suggests that ErbB/AKT/ERK signaling pathway may be potent biomarkers for PTX sensitivity and potential therapeutic targets for gastric cancer treatment.

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**Disclosure of conflict of interest**

None.

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## AKT/ERK is associated with GC resistance to paclitaxel

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## AKT/ERK is associated with GC resistance to paclitaxel

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