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Genes Correlated with Gemcitabine Efficacy in Non-small Cell Lung Cancer

Chunhong Li[1], Meiyan Liu[1], Hailing Lu[1], Wei Liu[1], Li Cai[1]* and Xiaoqun Dong[2]*

[1]*Department of Internal Medical Oncology, The Harbin Medical University Cancer Hospital, Harbin, P.R. China*
[2]*Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, The University of Rhode Island, Kingston, USA*

Abstract

Objective: Gemcitabine in combination with platinum improves survival of patients with non-small cell lung cancer (NSCLC). The purpose of the study was to explore genes related to gemcitabine efficacy.

Methods: The sensitivity of NSCLC cell lines to anticancer drugs was tested via MTT assay. Gene expression analysis was performed by cDNA microarray, and qRT-PCR was used for verification of the microarray results on highly sensitive genes. Fluorouracil (5-Fu) was used as the negative control of gemcitabine.

Results: Gemcitabine-related and fluorouracil-related genes were pooled into different clusters. Genes negatively related to 5-Fu sensitivity were positively related to gemcitabine efficacy. Metallothionein, Cathepsin B, TIMP1 and Galectin-1 genes which were resisted to certain anticancer drugs were sensitive to gemcitabine ($P<0.05$).

Conclusion: Metallothionein, Cathepsin B, TIMP1 and Galectin-1 can be considered as the predictors for gemcitabine sensitivity.

Keywords: Gemcitabine; Chemotherapy sensitivity; Efficacy-related genes; Non-small cell lung cancer

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer related death worldwide [1]. NSCLC is any type of epithelial lung cancer other than small cell lung carcinoma (SCLC). The most common types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. NSCLC is relatively insensitive to chemotherapy compared to SCLC. Platinum-based combination chemotherapy is the standard treatment for NSCLC [2], including cisplatin plus gemcitabine [3], vinorelbine [4], paclitaxel [5], docetaxel [6] or pemetrexed [7]. Cisplatin plus gemcitabine is currently one of the most applied regimens, with an overall response rate of 30%–38% [8,9]. A meta-analysis on 13 randomized trials demonstrates that gemcitabine shows a better survival than other anticancer drugs in advanced NSCLC, and a significant decrease in disease progression risk and overall mortality is found in gemcitabine-platinum regimen, with an absolute 1-year survival benefit of 3.9% [10]. It is reported that different outcomes of lung cancers are determined by different genetic phenotypes [11]. The study aims to identify which genes are related with gemcitabine efficacy in NSCLC.

Materials and Methods

Cell culture and drugs

Six NSCLC cell lines (LK-2 [squamous cell carcinoma], PC-7 [adenocarcinoma], PC-9 [adenocarcinoma], PC-14 [adenocarcinoma], A549 [adenocarcinoma] and Lu65 [large cell carcinoma]) and BET2A were cultured in RPMI 1640 supplement with 5% fetal bovine serum at 37°C in humidified air containing 5% CO_2. Gemcitabine was purchased from Lilly (America) and its concentration was adjusted to 0.05 μg/mL to 500 μg/mL. 5-Fu was purchased from Faulding (Australia) and its concentration was adjusted to 0.005 μg/mL to 10 μg/mL with dimethylsulphoxide (DMSO).

Measurement of cell sensitivity

Drug sensitivity was assessed by MTT assay [12]. Cells were seeded in 96 well tissue culture plates at an initial concentration of 1×10^5 cells/ml and pre-treated with different concentrations of gemcitabine (500, 100, 50, 10, 5, 1, 0.2, 0.1, 0.04 μg/ml) or 5-FU (10, 5, 1, 0.2, 0.1, 0.01, 0.04, 0.001, 0.008 μg/ml) for 68 h. Then cells were treated by addition of 20 ml MTT dye to each well. After incubation for 4 h, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with 200 μl DMSO in isopropanol. The absorbance was measured at 560 nm and the cell survival ratio was expressed as a percentage of the control. The IC_{50} was calculated using Reed-Muench method [13].

mRNA extraction and labelling

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). The mRNA was obtained with oligo-dT-magnetic beads (Toyobo Co, Osaka, Japan). For cDNA synthesis, River Trace (Toyobo Co, Osaka, Japan) was used. Probes were synthesized and labelled from 4 μg of amplified RNA. In brief, 4 μg of amplified RNA were combined with 4 μg amine-modified random primer and 5 units of RNAase inhibitor (SUPERase, Ambion). The mixture was incubated at 70°C for 10 min, then chilled on ice for 10 min, and left at room temperature for 10 min. Primer RNA solution was added to the reverse transcriptase mix (including 0.5 mM dATP, dGTP, dCTP, 0.3 mM dTTP, and 0.2

***Corresponding author:** Li Cai, Department of Internal Medical Oncology, The Harbin Medical University Cancer Hospital, Harbin, 150040, P.R. China, Tel: +86-451-86298283; Fax: +86-451-86298735; E-mail: caili366@126.com

Xiaoqun Dong, M.D., Ph.D., Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, The University of Rhode Island, Pharmacy Building, 7 Greenhouse Road, Kingston, RI 02881, USA, Tel: +1 401-874-4805; Fax: +1 401-874-5787; E-mail: xiaoqun_dong@uri.edu

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mM aminoallyl-dUTP) and incubated at 42°C for 2 h. The reaction was terminated by adding 10 µl 0.5 M EDTA, and RNA was hydrolysed with 10 µl 1 M NaOH at 65°C for 30 min.

cDNA array hybridization

Filters were pre-incubated in 30 ml of Perfect Hyb (Toyobo Co, Osaka, Japan) at 68°C for 30 min. Biotin-labelled probes (100 µl) were denatured at 100°C for 5 min. The filters were incubated in the hybridization mixture at 68°C for 20 h, and the filters were washed 3 times at 68°C for 10 min with 30 ml 2 × SSC/0.1% sodium dodecyl sulfate (SDS) and 0.1 × SSC/0.1% SDS. Signals were detected with the Imaging High Chemifluorescence Detection Kit (Toyobo Co, Osaka, Japan). Vistra ECF substrate (AttoPhos) (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as the chemifluorescence substrate. Substrate images were acquired by Fluorimager (Amersham Pharmacia Biotech, Uppsala, Sweden).

Data analysis

"H" (high) and "L" (low) was defined according to the grey degree in lung cancer cell lines and in BET2A cell lines respectively. Gene expression was considered as up-regulation if H/L>3.0, and down-regulation if H/L<0.333. "A" (CI50) represented drug activity; "T" was the gene expression data including the radio of expression intensity of genes and BET2A cells. The AT-clustered image map (CIM) summarizes the relationship between drug activity and gene expression. In this case, drugs clustered together with related genes, and genes were also clustered together with related drugs. Colour reflected the connection between gene and drug (red meant that the gene was sensitive to the drug with the high positive correlation, blue with the high negative correlation, yellow or green with the lower correlation). Difference was considered statistically significant when a p value was <0.05 (Pearson correlation coefficient ≥ 0.632, P<0.05; Pearson correlation coefficient ≥ 0.715, P<0.02).

RT-PCR

Total RNA was extracted from 6 cell lines according to the manufacturer's instructions. RT-PCR was performed in 50 µL reactions using 100 ng of RNA, 0.5 µmol/L of each primer. All other PCR conditions and reagents were supplied and recommended by the manufacturer's protocol for the Titan one-step system (Roche Applied Science, Indianapolis, IN, USA). Beta-actin served as an internal control. Cycling conditions: beta-actin: 23 cycles at 94°C for 3 min, 94°C for 20 s, 56°C for 30 s, 72°C for 50 s, and 72°C for 10 min. Metallothionein and TIMP-1: 32 and 22 cycles respectively at 94°C for 3 min, 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, and 72°C for 10 min. The forward primer for Metallothionein was 5'-TCTCACCTCGGCTTGCAATGGAC and the reverse primer was 3'-ACAGCAGGGCTGTCCCGACATC. The forward primer for TIMP-1 was GACCTCGTCATCAGGGCCAAGTTC and the reverse primer TTCAGAGCCTTGGAGGAGCTGGTC. The forward primer for β-actin was 5'-AGCGCAAGTACTCCGTGTG and the reverse primer was 3'-AAGCAATGCTATCACCTCC. The amplification products were visualized by bromide-ethyl pastille following sodium dodecyl sulphate–polyacrylamide gel electrophoresis with the DL-2000 molecular weight standard (Gene Runner).

Results

Growth inhibitory activities (IC_{50}) of anticancer agents against lung cancer were shown in Table 1. The connection was analysed between gene expression and anti-cancer drugs activity in 6 NSCLC cell lines. Gene expression profiling data related to the activity of gemcitabine and 5-Fu was shown in Figure 1. The profile of gene expression was clustered well, and high consistency of gene expression existed in gemcitabine-group as well as in 5-Fu-group. Moreover, activity-related genes in gemcitabine-group were gathered into a cluster, while genes in the 5-Fu-group came together into another cluster. The activity-related genes with gemcitabine were mainly divided into the following 5 categories: Signal transduction molecules, growth factor, growth factor receptors, apoptosis cascade and transcription factors (Table 2).

36 genes were related with gemcitabine activity. Most genes positively related with gemcitabine activity were negatively related to 5-Fu activity. Among genes differentially expressed, Metallothionein, Cathepsin B, TIMP1 and Galectin-1 were highly positively associated with the sensitivity of gemcitabine (P<0.05) (Table 3). Drug activity-related genes Metallothionein and TIMP-1 were selected to test microarray data by semi-quantitative RT-PCR (Figure 2). The result of RT-PCR was in consistent with cDNA microarray data.

Discussion

Chemotherapy is a crucial treatment against lung cancer. The same chemotherapeutic medicine is frequently used for patients with different types of cancer. Meanwhile, the different anticancer drugs are applied in patients with the same type of cancer. It is due to certain genes existed in tumours that respond to certain drugs. Therefore, it is important to find out drug sensitivity-related genes in choosing an effective chemotherapy regimen. Much effort has been put on the relation between resistant genes and anticancer drugs. Patients with an increased expression of excision repair cross complementation group-1 (ERCC1) or ribonucleotide reductase subunit M1 (RRM1) may benefit less from cisplatin-based and gemcitabine-based chemotherapy, respectively [14]. Overexpression of P-glycoprotein is associated with taxanes resistance. Clinical studies support a relationship between

Cell Lines	IC50 (µg/ml)	
	GEM	5-Fu
Lu65	79.533 ± 10.854	8.759 ± 1.007
LK-2	54.607 ± 7.989	>10
PC-9	14.210 ± 3.571	5.843 ± 2.239
PC-7	<0.050	2.760 ± 1.174
PC14	>500	>10
A549	>500	>10
*GEM: Gemcitabine; 5-Fu: 5-Fluorouracil		

Table 1: Growth inhibitory activities (IC50) of anticancer agents against the lung cancer cell lines.

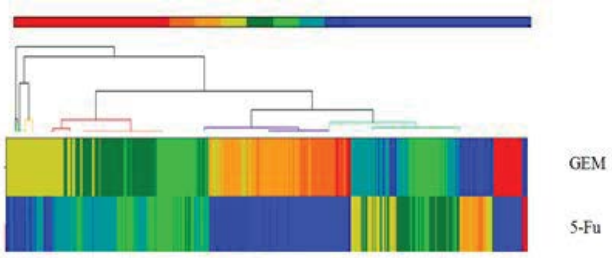

Figure 1: Clustered image map (CIM) in the relation between drug activity and gene expression in NSCLC cell lines. Red meant that the gene was sensitive to the drug with the high positive correlation, blue with the high negative correlation, yellow or green with the lower correlation. Activity-related genes in gemcitabine-group were gathered into a cluster, while genes in the 5-Fu-group came together into another cluster.

Classification	GEM (Gene Number)	5-Fu (Gene Number)
Signal transduction molecule	11	11
Growth factor receptor	4	4
Growth factor	6	6
Apoptosis related	2	2
Cell factor	1	1
Cyclin protein	1	1
Transcription factor	1	1
Metabolism-related enzymes and inhibitors	1	1
Proteolysis	1	1
Molecular chaperone	1	1
cell surface receptor	0	0
Development process factor	0	0
Others*	7	10
Total	36	39
*Refers to non-classified genes		

Table 2: Classification of sensitive gene to gemcitabine (GEM) and 5-fluorouracil (5-Fu) in 6 lung cancer cell lines.

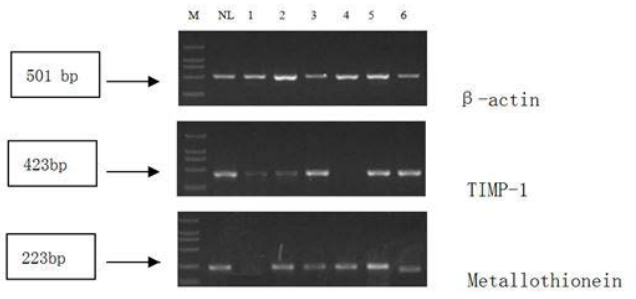

Figure 2: TIMP-1 and Metallothionein expressed in 6 NSCLC cell lines by RT-PCR. M: Mark, NL: Normal lung cell. 1-6 represented PC-7, LK-2, PC-9, LU65, A549, and PC-14 cell lines respectively. **(A)** PCR product of β-actin, as an internal control; **(B)** TIMP-1 expressed weakly in PC-7 and LK-2 cell lines; **(C)** Metallothionein expressed weakly in PC-7, PC-9, LK-2 and LU65 cell lines.

poor response to taxanes and overexpression of beta III-tubulin [15]. Phosphohydroxythreonine aminotransferase (PSAT1) was related with less response of oxaliplatin treatment [16]. However, most studies focused on the association between single gene and single drug in cancer. If several markers are detected in combination, it will provide the better prediction for treatment response.

Gemcitabine and 5-Fu are anticancer drugs acting on cancer metabolism [17,18]. Gemcitabine (dFdC) [19] is phosphorylated into gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK), and subsequently phosphorylated to gemcitabine diphosphate (dFdCDP) by pyrimidine nucleoside monophosphate kinase and gemcitabine triphosphate (dFdCTP) by nucleoside diphosphate kinase (NDPK). dFdCTP is incorporated into DNA during replication, and inhibited chain elongation of DNA and cause cell apoptosis. Gemcitabine is rapidly metabolized by cytidine deaminase in liver, kidney, blood and other tissues. A half-life (t1/2) of Gemcitabine is 30 to 90 minutes associated with age and sex. Fluorouracil [20] is transformed into a 5-fluoro-2-deoxyuracil nucleotide, which inhibit thymine nucleotide synthetase, block Deoxyuracil nucleotide into Deoxythymine nucleotide and inhibit the biosynthesis of DNA. Fluorouracil is mainly metabolized by the liver, and is decomposed into carbon dioxide, about 15% of prototype medicine out of the body by kidney. Large doses of the drug can pass through the blood-brain barrier and reach the cerebrospinal fluid after intravenous infusion for

Entering serial number	Genes	GEM	5-Fu
X64177	Metallothionein	0.731	-0.734
L16510	Cathepsin B	0.715	-0.723
X03124	TIMP1	0.700	-0.692
J04456	Galectin-1	0.695	-0.702
X55313	TNF-R1	0.621	-0.636
*	TGF, beta-induced, 68KD	0.572	-0.596
X12451	Cathepsin L	0.550	-0.566
M16006	PAI-1	0.503	-0.525
*	Annexin 11	0.506	-0.530
M62403	IGFBP4	0.574	-0.588
X51675	UPAR	0.493	-0.508
U61276	Jagged	0.426	-0.450
U03864	Alpha A-AR	0.585	-0.582
M59371	EphA2	0.525	-0.543
X13276	CD13	0.456	-0.470
U66075	GATA-6	0.441	-0.466
M74088	APC	0.421	-0.433
*	Fibromodulin	0.431	-0.454
AB002409	SLC	0.400	-0.420
M14113	Procoagulant	-0.45	0.421
M64722	Clusterin	0.610	-0.632
U20240	C/EBP gamma	0.412	-0.435
J04456	HSP32	0.451	-0.471
Y00371	HSC 70	0.532	-0.548
L25081	Rho C	0.473	-0.487
M87770	FGFR-2	0.542	-0.550
X14787	Thrombospondin 1	0.516	-0.517
M33680	CD81	0.406	-0.435
*	Thymosin beta 10	0.412	-0.436
U22322	Rak	0.415	-0.426
*	Lactate dehydrogenase A	0.416	-0.431
U01877	P300	0.419	-0.436
X61615	LIFR	0.403	-0.422
Z12020	MDM2	-0.435	0.417
AF101264	CaMKK	-0.407	--
J03817	GSTM1B	--	-0.401
M15518	TPA	-0.413	--
J04765	CD29	--	-0.408
X15804	Osteopontin	--	-0.410
L20688	Alpha-actin	--	-0.409
*	Rho GDI beta	--	0.415
*Refers to non-classified genes. GEM: gemcitabine. 5-Fu: 5-fluorouracil. The number means Pearson correlation coefficient. Pearson correlation coefficient ≥ 0.632, P<0.05; Pearson correlation coefficient ≥ 0.715, P<0.02			

Table 3: Genes related with drug activity of gemcitabine and 5-fluorouracil in NSCLC.

half an hour, lasting 3 hours. t1/2α is 10-20 minutes, and t1/2β is 20 hours. Although it is similar to gemcitabine in antitumor mechanism, 5-Fu is rarely applied in NSCLC treatment, while gemcitabine frequently appears in chemotherapy regimens against NSCLC [21].

Conclusion

Therefore, 5-Fu was used for a negative control to gemcitabine in the study. The study data showed that genes positively connected with gemcitabine were mainly negatively connected with 5-Fu, which can be a reason for the lack of 5-Fu in NSCLC treatment. Metallothionein, Cathepsin B, TIMP1 and Galectin-1 were highly positively associated

with the sensitivity of gemcitabine. The four genes can be considered as gemcitabine efficacy-related genes which may be applied clinically to predict the response of gemcitabine in NSCLC. To gemcitabine-insensitivity patients, gemcitabine should be exclude in treatment regimen, and avoid the adverse effects including difficulty breathing, low white and red blood cells counts and low platelet counts, vomiting and nausea, elevated transaminases, rashes and itchy skin, hair loss, blood and protein in urine, flu-like symptoms, edema, fever, loss of appetite, headache, difficulty sleeping, tiredness, cough, runny nose, diarrhoea, mouth and lip sores, sweating, back pain, and muscle pain.

Metallothionein (MT) expression level is related with drug resistance in a variety of malignancies including NSCLC. MTs play important roles in the resistance of tumour cells to cisplatin [22]. Cathepsins B (CTSB) is involved in tumorigenesis, angiogenesis, invasion and metastasis [23]. Over-expression of CTSB is correlated with poor prognosis and increases incidence of distant metastases. CTSB is connected with drug resistance [24]. TIMP-1 influences cell growth and apoptosis [25]. TIMP-1 levels were significantly associated with a poor response to chemotherapy in patients with metastatic breast cancer, and TIMP-1 is resistant to the most frequently used chemotherapy regimens of cyclophosphamide/methotrexate/5-Fu [26]. Galectin-1 knockdown sensitized lung cancer cells to platinum-based chemotherapy (cisplatin) [27]. The above-mentioned genes which are reported resistant to certain anticancer drugs are unexpectedly sensitive to gemcitabine in our study. The observation provided theoretical evidence in explaining why gemcitabine produces a good survival benefit against other agents in the combination with platinum. In fact, it has been reported that gemcitabine can increase the sensitivity of both cisplatin-sensitive and cisplatin-resistant cell lines [28].

Future medication will be tailored based on the individual's genetics. A number of potential biomarkers are under investigation with an attempt to provide optimal therapies. The results can provide potential biomarkers for the prediction of gemcitabine efficacy and afford potential targets to overcome gemcitabine resistance in NSCLC patients.

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