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In vitro Simulation Study of Individualized Chemotherapy in Lung Cancer

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Abstract

The primary aim of this *in vitro* simulation study was to evaluate the utility of gene expression profile analysis in predicting the effect of varying drug combinations for the treatment of lung cancer. Using 10 human cancer cell lines, we focused our gene expression analysis on a cohort of candidate sensitivity-prediction factors, previously reported using cDNA filter arrays, with a view to predicting the ability of a set of anti-cancer drugs commonly used to treat lung cancer, namely cisplatin, 5-fluorouracil (5FU), SN38, docetaxel, gemcitabine, and vinorelbine. Altered expression of genes for glutathione-S-transferase-pi, uridine phosphorylase, O-6-methylguanine-DNA methyltransferase, and multidrug resistance 1 was identified in lung cancer cell lines. Drug sensitivity testing, in the form of methylthiotetrazol analysis, was performed using these six anti-cancer drugs against the panel of 10 lung cancer cell lines. We compared the predicted chemosensitivity based on the gene expression pattern of 19 well-known sensitivity-related genes with the cytotoxic activity of each of these anti-cancer drugs. Molecular profiling data predicted resistance to CDDP in LK-2 cells, 5FU in LK-2, PC7, A549, NCI-N231, Lu135 cells, irinitecan in PC9 cells, and VNR in PC7 cells. However, the prediction efficacy (number of predicted inactive drugs by gene expression analysis/number of inactive drugs by methylthiotetrazol assay) was 21.6% (8 of 37). No false-positive findings in relation to sensitivity-related genes were obtained on the basis of this molecular analysis. Thus, prediction of sensitivity to lung cancer by molecular analysis appears possible. With elucidation of additional drug sensitivity factors, selection of appropriate anticancer drugs by gene expression profiling may make it possible to increase the response rate in lung cancer chemotherapy.
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Key words: taylor-made chemotherapy, cDNA array, lung cancer, cell lines

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Introduction

Patients with solid tumors still have a poor prognosis, in spite of the development of a wide range of anticancer agents. Many theories have been developed about the optimization of cancer chemotherapy, including the use of more intensive treatment schedules[1,2] and alternative chemotherapy[3,4] and the application of relevant sensitivity tests *in vitro*[5,6]; however, significant improvement is still needed in this area. With regard to the treatment of lung cancer, there are many anticancer agents in use, such as platinum compounds (such as cisplatin and carboplatin), taxanes (docetaxel and paclitaxel), vinorelbine, gemcitabine, 5FU, irinotecan. A number of combination therapy regimens employing platinum compounds have proven to be quite effective and are widely applied to the treatment of inoperative non-small cell lung cancer[7,8]. Furthermore, there are many ongoing clinical trials that may further refine the use of these agents for the treatment of non-small cell lung cancer[9–12]. However, the effect of these therapies on improving patient survival remain far from satisfactory[7,8,13]. Empirical therapy for lung cancer seems inadequate, principally due to genetic heterogeneity and individual differences in relation to chemosensitivity to anticancer drugs. It is consequently desirable to find more appropriate therapeutic opportunities based on acquired insights. Many intrinsic-resistance mechanisms to various anti-cancer drugs have been reported for the purpose of the individualization of cancer chemotherapy.

The primary aim of this study was to evaluate the utility of gene expression profiling for selecting appropriate drugs to be used in the treatment of lung cancer. Using 10 human cancer cell lines, we performed DNA array-based gene expression analysis using complementary (c) DNA filter arrays. We focused our analysis on the expression of 19 well-known sensitivity-related genes and predicted drug sensitivity to 6 commonly used anticancer agents, cisplatin, 5-fluorouracil (5-FU), SN38, docetaxel, gemcitabine, and vinorelbine. SN38 is an active form of irinotecan. In addition, we examined the sensitivity of these cell lines to the 6 anticancer agents by methylthiotetrazol (MTT) assay. We then compared the predicted sensitivity with the actual cytotoxic activity of each of the anticancer agents by MTT assay in all the cell lines.

Materials and Methods

Cell Lines

The PC7, PC9, and PC14 cell lines were obtained from IBL (Gumma, Japan). The A549, NCI-H69, and NCI-N231 cell lines were obtained from the American Type Culture Collection (Rockville, MD)[14]. The Lu65 and Lu135 cell lines were provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan)[14]. The LK-2 and SBC-3 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). PC7, PC9, PC14, A549 and Lu65 are lung adenocarcinoma cell lines. LK-2 is a squamous cell lung cancer cell line. NCI-H69, NCI-N231, Lu135, and SBC-3 are small cell lung cancer cell lines.

RNA Isolation, cDNA Array Hybridization and Analysis of Hybridization Signals

Total RNA was isolated from each cell line using standard protocols as described previously[14,15]. Messenger (m) RNA was then purified from total RNA by incubation with oligo-dT-magnetic beads (Toyobo Co., Osaka, Japan)[14,15]. The ElectorGene Array System (GeneticLab. Co., Ltd. Sapporo, Japan) was used for filter-based cDNA array analysis, as previously reported[14,15]. Thirteen hundred individual human DNA fragments were spotted in duplicate on a filter. The genes represented on this array included various cancer-related and drug resistance-associated genes, as well as housekeeping and nonmammalian genes as controls. To prepare the probes, reverse transcription was performed using Reverse Transcriptase, ReverTraAce (Toyobo Co., Osaka, Japan), together with a random 9-mer (Toyobo Co., Osaka, Japan) as the primer and 5 μg of polyA RNA. The probes were labeled with biotin by incorporation of biotin-16-deoxyuracil triphosphate during the synthesis of cDNA. The filters were

preincubated in 20 m*l* of PerfectHyb (Toyobo Co., Osaka, Japan) at 68℃ for 30 minutes. The biotin-labeled probes were denatured and added to the prehybridization solution. The filters were incubated overnight at 68℃ in the hybridization mixture. After washing, specific signals on the filters were detected with the Imaging High - Chemilumi - Detection kit (Toyobo Co., Osaka, Japan). CDP-Star (Tropix, Bedford, MA) was used as the chemiluminescence substrate. A chemiluminescence image of the filter was acquired by Fluor-S (Bio-Rad, Hercules, CA). Gene expression levels were quantified by measuring the intensity of the signals using Imagene (BioDiscovery, Los Angeles, CA). The signal intensity among filters was analyzed by the ElectorGene Finding System (GeneticLab, Sapporo, Japan). The background threshold was set at a 3-fold higher level than the negative control. Signal intensities were normalized against the expression data relating to housekeeping genes, namely comparing the expression of the housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin.

MTT Assay

Cytotoxicity in the cell lines was estimated with a rapid colorimetric assay for mitochondrial dehydrogenase activity, as previously described[14]. Briefly, cells were seeded into 12-well plates (Falcon, Lincoln Park, NJ). Following 24 hours' exposure to particular anticancer agents, the cells were washed twice and incubated for a further 24 hours in drug-free medium. The cells were then incubated with 0.5 mg/mL MTT for 4 hours. The blue formazan crystals, formed by viable cells, were solubilized by the addition of 10% n-dodecylsulfate sodium salt in 0.01N HCL, followed by overnight incubation. Samples were then subjected to spectrophotometric analysis at 560 nm (Ultraspec 4050; LKB, Bromma, Sweden).

Results

Nineteen chemosensitivity-prediction factors, identified from previous reports, were selected for this *in vitro* simulation study of individualized chemotherapy in lung cancer (**Table 1**). For gene expression profiling purposes, we used filter-based DNA arrays, representing 1,300 cancer-related and drug-resistance-associated genes, to examine responses in a panel of human lung cancer cell lines exposed to different drug combinations (**Fig. 1**). To avoid the potentially conflicting influence of cell culture conditions, we separately cultured each cell line in 6 bottles[15]. On the array, probes for the controls, such as GAPDH, β-actin genes, were located at the outer line in the opposite angle in duplicate. A standard curve was obtained with reference to serially diluted spots of GAPDH. The expression level of each gene was calculated by comparison with an internal standard. We then focused our analysis on the expression data relating

Table 1 Genes reported to be linked with drug resistance

Drug	Gene	Expression level in cells with drug resistance
CDDP	ERCC 1	increased
	c-moat	increased
	gamma-GCS	increased
	GST-pi	increased
	metallothionein	increased
5FU	DPD	increased
	TS	increased
	OPRT	decreased
	uridine phosphorylase	decreased
	uridine kinase	decreased
	MRP	increased
CPT-11	MGMT	increased
	topo-1	increased
	gamma-GCS	increased
DOC	beta-tubulin	altered
GEM	cytidine deaminase	increased
	deoxycytidine kinase	decreased
	ribonucleotide reductase	increased
VNR	MRP4	increased
	MDR1	increased

ERCC: excision repair cross-complementation, c-moat: canalicular multispecific organic anion transporter, GCS: glutamylcysteine synthetase, GST: glutathione-S-transferase, DPD: dihydropyrimidine dehydrogenase, TS: thymidylate synthase, OPRT: Orotate Phosphoribosyl-Transferase, MRP: multidrug resistance-associated protein, UP: uridine phosphorylase, MDR: multidrug resistance, MGMT: O-6-methylguanine-DNA methyltransferase.

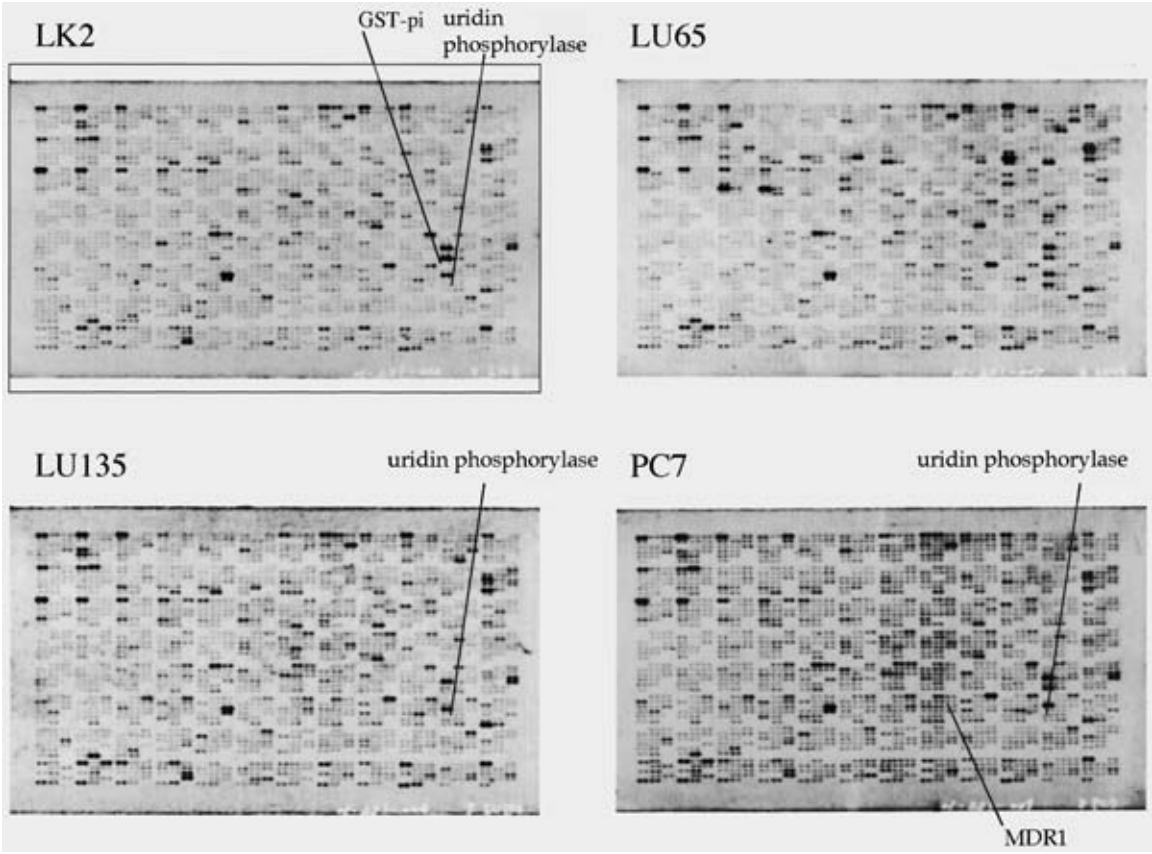

Fig. 1 DNA array-based expression profiling of cancer-related and drug sensitivity-related genes in LK2, Lu65, Lu135, and PC7 cells using the ElectorGene Array System (GeneticLab. Co., Ltd. Sapporo, Japan).

to the preidentified set of chemosensitivity-prediction factors. **Table 2** shows the expression status of these selected chemosensitivity-prediction factors. The differences in expression levels that were either >2.5 or <0.4 fold in nature were deemed significant. Altered expressions of glutathione-S-transferase (GST)-pi, uridine phosphorylase, O-6-methylguanine-DNA methyltransferase (MGMT), and multidrug resistance (MDR) 1 genes were identified in lung cancer cell lines. Drug sensitivity testing, namely by MTT analysis, was performed on the 10 lung cancer cell lines. Six anticancer drugs currently used for lung cancer chemotherapy; namely namely cisplatin, 5-FU, SN38, docetaxel, gemcitabine, and vinorelbine were selected for our analyses. When the observed median inhibitory concentration was lower than the Cmax in clinical usage (cisplatin 4 ug/m*l*, 5FU 0.5 ug/m*l*, SN-38 0.1 ug/m*l*, docetaxel 4 ug/m*l*, gemcitabine 15 ug/m*l*, vinorelbine 0.1 ug/m*l*), the analyzed drug was classified as being "effective".

We compared the predicted chemosensitivity based on the gene expression of 19 well-known sensitivity-related genes with the observed cytotoxic activity of each of these anticancer drugs *in vitro*. The overall results are summarized in **Table 2**. Resutls of molecular profiling predicted resistance to cisplatin in LK-2 cells, 5-FU in LK-2, PC7, A549, NCI-N231, and Lu135 cells; to irinotecan in PC9 cells; and to vinorelbine in PC7 cells. Eight drugs were predicted to be inactive in this set of lung cancer cell lines. However, 37 drugs were found by MTT assay to be inactive in this panel of cell lines. The prediction efficacy (the number of predicted inactive drugs by gene expression analysis/the actual number of inactive drugs by MTT assay) was 21.6% (8 of 37). The sensitivity of this method for determining the resistance to these anticancer drugs was as follows; cisplatin, 25%; 5-FU, 55.6%; irinotecan, 12.5%; docetaxel, 0%; gemcitabine, 0%; and vinorelbine, 20%. No false-positive findings in 18 other factors were detected by this molecular

Table 2 Predicted chemosensitivity based on the expression of sensitivity-related genes and the cytotoxic activity of selected anticancer drugs in lung cancer cell lines

Cell lines	Expression analysis	Predicted inactive drugs by gene expression	Sensitivity test (MTT assay)					
			CDDP	SN-38	VNR	DOC	GEM	5FU
LK-2	GST-pi (up), UP (down)	XCDDP X 5FU	X	X	O	X	X	X
PC7	MDR (up), UP (down)	X VNR X 5FU	X	X	X	O	O	X
PC9	MGMT (up)	X CPT-11	O	X	X	X	O	X
PC14	No alteration	OAll	O	X	X	X	X	X
A549	UP (down)	X 5FU	X	X	X	X	X	X
Lu65	No alteration	OAll	O	X	O	O	X	X
SBC-3	No alteration	OAll	X	O	X	X	O	O
NCI-H69	No alteration	OAll	O	X	O	X	O	X
NCI-N231	UP (down)	X 5FU	O	X	O	X	O	X
Lu135	UP (down)	X 5FU	O	O	O	O	O	X

O: active, X: inactive, GST: glutathione-S-transferase, UP: uridine phosphorylase, MDR: multidrug resistance, MGMT: O-6-methylguanine-DNA methyltransferase.

profiling approach.

Discussion

In this *in vitro* simulation study, we used a DNA array-based gene expression approach, together with assessment of the cytotoxic activity of several widely applied anticancer agents, in a collection of human lung cancer cell lines. We used filter-based DNA arrays, representing 1,300 cancer-related and drug-resistance-associated genes, for gene expression profiling. We then analyzed the gene expression data of selected chemosensitivity-prediction factors. Altered gene expressions of several chemosensitivity-prediction factors were identified in lung cancer cell lines.

In particular, altered expressions of GST-pi, uridine phosphorylase, MGMT, and MDR1 genes were found. We compared the predicted chemosensitivity based on these gene expression profiles with the cytotoxic activity of each of these anticancer drugs as determined by MTT assay. Resistance to cisplatin, 5-FU, irinotecan, and vinorelbine were predicted with this molecular profiling approach. However, prediction efficacy was 21.6% (8 of 37). This is a first report published of an *in vitro* simulation study of lung cancer chemotherapy using well-known chemosensitivity-prediction factors on a cDNA array. In previous chemosensitivity-prediction studies, only a single or a few factors were evaluated. However, the sensitivity of this approach using a cDNA array seems to be too low. Previous studies evaluating a single factor generally showed altered expression in the tumors more frequently than did our study[16–18]. A possible reason for these apparent differences may be due to the different analysis techniques used in these studies. Many studies analyzing chemosensitivity prediction factors have been performed at the protein level, with Western blot analysis or immunohistochemical techniques or both.

According to our study, molecular analysis is able to predict a response to chemotherapy in lung cancer, although the method is not completely satisfactory. Appropriate selection of anticancer drugs on the basis of gene expression profiling data may, in the future, make it possible to elevate response rate in patients receiving chemotherapy for lung cancer. However, because gene expression profiling using cDNA arrays is expensive, the sensitivity of this approach should be increased by discovering additional prediction factors and by

improved systems for monitoring alterations in gene expression. In the future, molecular profiling is expected to provide significant benefits to individualized therapy.

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