Identification of Genes Induced by Taxol Application Using a Combination of Differential Display RT-PCR and DNA Microarray Analysis

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The differential display reverse transcriptional polymerase chain reaction (DD-RT-PCR) was used to hunt for cDNA fragments specifically expressed by taxol treatment of HeLa cells. Forty-eight cDNA clones were differentially displayed through the experiments. The cDNA fragments obtained were separately spotted onto glass slides to prepare a tailor-made DNA chip. The gene expression pattern of differentially displayed cDNA fragments were checked by DNA microarray analysis.

Introduction

A mitotic inhibitor suppresses the active cells proliferation, which is the most characteristic property of cancer cell. Mitotic inhibitors can be classified into two groups by their mode of action. The first involves reagents that directly act on mitotic machinery, and the second group are reagents that affect the intermediary metabolism causing a inhibition of cell division. The drug taxol used in this study, belongs to the former group. Microtubules are the vital element forming the spindle structure which is the main mitotic machinery in eukaryotic cells, and thus become a target for inhibitors of cell division (Niitani and Hisakatsu, 1993) Colchicine and *Vinca* alkaloids are known as compounds acting on microtubules directly, and their mode of action is inhibition of microtubule polymerization. (Andreu and Timasheff, 1982; Wilson *et al.*, 1975) Taxol, which has been attracting attention as an anti-cancer reagent in the clinical field, has a unique mode of action that involves a promotion of microtubule polymerization, and obstruction of its depolymerization (Wani *et al.,* 1971) Taxol directly affects microtubules; in addition, it has a completely different effect that inhibits the function of an apoptosis restraining protein "bcl-2″, to finally induce apoptosis (Liu *et al.,* 1994). At present, tough research on the microtubule taxol binding site and the elucidation of the induction mechanism of apoptosis by taxol is widely carried out, but the whole process has not yet been resolved.

We searched for cDNA fragments specifically expressed by taxol treatment, expecting this study would assist the elucidation of mode of taxol action. HeLa cells were used as model cancer cells, and the "differential display reverse transcriptional PCR" technique (DD-RTPCR) was employed for hunting specific gene fragments. (Liang and Pardee, 1992) The new technique, "DNA microarray method" was performed as a tool to verify the obtained gene expression (Schena *et al.,* 1995).

Materials and Methods

Cell and cell culture

HeLa Cells (clone RCB0007) were obtained from RIKEN Cell Bank, Institute of Physical and Chemical Research (RIKEN) (Ibaraki Japan) and maintained by routine replacement of fresh medium according to the conditions described below. Cell lines was cultured at 37 °C in a medium consisting of MEM, which is Dulbecco's modified Eagle medium (Dainippon Pharm. Co., Osaka, Japan), supplemented with sodium bicarbonate

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(final conc.: 0.06%), L-glutamate (final conc.: 2 mM), penicillin (final conc.: 50 IU), streptomycin (final conc.: 50 ml/ml) and 10% fetal calf serum (ICN Pharmaceutical, Inc., Costa Mesa, CA, USA). Cultures medium were routinely replaced by fresh medium. Cell viability was evaluated by the crystal violet staining method.

Differential display RT-PCR(DD-RT-PCR)

Taxol was administrated to HeLa cell cultures one day after medium replacement at conc. of 117 nM for three hours. Cells were harvested by scraping. Total RNA from harvested cells was isolated using Quick Prep RNA extraction kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). Differential display RT-PCR (DD-RT-PCR) was carried out between RNA from taxol-treated HeLa cells and non-treated HeLa cells according to the previously reported procedures. (Yoshida 1994) For DD-RT-PCR, 240 sets of arbitrary primers (BEX Co., Ltd, Tokyo CJapan) were employed. DD-RT-PCR products were subjected to 1.5% agarose gel electrophoresis to compare amplified products generated from the taxol-treated group with the non-treated group.

Cloning of cDNA fragments and DNA sequencing

The differentially displayed cDNA fragments were subcloned in pT7Blue T-vector (Novagen,-Inc., Madison, WI, USA) by conventional TA cloning methods. After purification of the plasmid DNA, each clone was sequenced with a model ABI Prism 310 genetic analyzer (PE Biosystems, Foster City, Calif., USA). Obtained sequence data were subjected to a homology search study by using the BLAST algorithm on network service. (URL: http://www.ddbj.nig.ac.jp/E-mail/homology-j.html)

DNA microarray analysis

The differentially displayed cDNA fragments amplified by PCR were spotted onto a slide glass using DNA chip preparation equipment(GMS417-arrayer, Takara Shuzo Co., Kyoto, Japan) to prepare the DNA microarray on which the differential displayed cDNA fragments are aligned. (Type of cDNA spotted and coordinates on the chip are described in the legend of Fig. 2.) mRNA (ca.2 mg) prepared from taxol-treated cell was reversely transcribed by AMV RTaseXL (Takara Shuzo Co., Kyoto, Japan) for labeling with fluorescent dye Cy5 (Molecular Probes, Inc., Eugene,OR, USA), incorporating Cy5-dUTP. According to a similar procedure, mRNA (ca.2 mg) prepared from non-treated cells was subjected to fluorescent labeling with Cy3-dUTP as a control. An equal amount of the two kind of samples were mixed and the resulting mixture was hybridized onto the above-mentioned DNA microarray. After washing and drying, the hybridized a DNA microarray was scanned by a DNA microarray analyzer(GMS418Array Scanner, Takara Shuzo Co., Japan). After scanning for each fluorescent dye separately, the red color was superimposed for Cy3 as a false color image and Cy5 was given a green as a false color image.

Results and Discussion

Evaluation of taxol-sensitivity of HeLa cell and acquisition of taxol tolerant cells

HeLa cell RCB0007 was innoculated to media containing various concentrations of taxol (0 to 1 ppm) to evaluate cell viability by the crystal violet staining method. The IC_{50} of HeLa cell RCB0007 against taxol was estimated to be 67 nM. (Fig. 1) In addition, a taxol-tolerant cell line was acquired by gradually rising the taxol concentration every sub culturing. IC_{50} of the tolerant cell obtained is estimated to be more than 5.1 nM (Fig. 1).

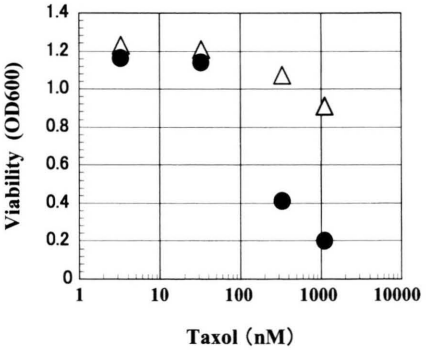

Fig. 1. Survival curve of HeLa cells and the taxol-tolerating HeLa cells against taxol.
HeLa Cells were obtained from RIKEN (clone RCB0007). Culture mediums were routinely replaced by fresh medium. Cell vitality was evaluated by the crystal violet staining method. Solid circles indicate HeLa cells and open triangles indicate taxol-tolerant HeLa cells.

Differential display analysis of mRNA species in taxol treated HeLa cells

We conducted the differential display RT-PCR (DD-RT-PCR) for acquiring the genes specifically expressed by taxol application according to the methods described above. The forty eight cDNA fragments obtained, are shown in Table I. The expression patterns of gene groups were analyzed by the DNA microarray method.

Expression analysis of differentially displayed genes

After scanning for each fluorescent dye separately, the red color was superimposed for Cy3 as a false color image and Cy5 was given a green false

Table I. Homology search results of differential displayed cDNA fragments.

Clone No.	Length(bp)	Homolgy	Identities	Score	Accession No.
A-1	319	Zinc finger protein 275	20/21 (95%)	36	U82670
A-2	388	CDK inhibitor p19INK4d mRNA	20/20(100%)	40	U40343
A-3	387	Genomic clone(not characterized)	19/19(100%)	38	AC005029
A-4	329	NONE	–	–	–
A-5	515	Genomic clone(not characterized)	24/24(100%)	48	AC004914
A-6	421	NONE	–	–	–
A-7	387	Translation initiation factor eIF-2	358/363(99%)	658	L19161
A-8	386	Sterol-C5-desaturase	368/390(94%)	527	AF069469
A-9	386	Type 3 collagen alpha 1.	379/386(98%)	680	E10600
A-10	257	Splicesomal protein (SAP 61)	246/250(98%)	442	U08815
A-11	257	Splicing factor SF3a60.	255/257(99%)	478	X81789
A-12	342	EST clone(not characterized)	296/305(97%)	539	AK000087
B-1	390	EST clone(not characterized)	371/376(99%)	676	AF131859
B-2	401	NONE	–	–	–
B-3	294	NONE	–	–	–
B-4	390	EST clone(not characterized)	371/376(99%)	676	AF131859
B-5	399	Huntington interacting protein (HIP2)	354/360(98%)	636	U58522
B-6	322	Cullin 4A (CUL4A)	318/324(98%)	581	AF077188
B-7	321	Thrombospondin.	298/304(98%)	545	X14787
B-8	384	Makorin 1 (MKRN1)	352/359(98%)	624	AF192784
B-9	514	EST clone(not characterized)	358/396(90%)	317	AK001611
B-10	392	KIAA1343 protein	373/389(96%)	624	AB037764
B-11	328	Human caldesmon	153/171(89%)	194	E05382
B-12	300	Human caldesmon	164/165(99%)	319	E05382
C-1	352	Chaperonin pseudogene	248/272(91%)	333	AL035634
C-2	388	Sopeptidase T-3	384/392(98%)	678	U75362
C-3	302	EST clone(not characterized)	189/190(99%)	371	AL050321
C-4	289	KIAA0694 protein	264/291(91%)	383	AB014594
C-5	308	NAD^+ ADP-ribosyltransferase (ADPRT)	134/135(99%)	262	M29785
C-6	241	EST clone(not characterized)	21/21(100%)	42	AC007750
C-7	387	KIAA0368 protein	343/351(97%)	587	AB002366
C-8	315	Zinc finger transcription factor (ZNF207)	231/236(98%)	398	AF046001
C-9	297	Protein kinase PRK2	284/290(98%)	535	S75548
C-10	323	Alpha 4 protein	321/324(99%)	605	Y08915
C-11	226	Myosin-I beta	180/187 (96%)	323	X98507
C-12	319	Myosin-I beta	313/319 (98%)	553	X98507
D-1	360	Furin	319/329 (96%)	567	E14286
D-2	325	EST clone (not characterized)	273/307 (88%)	242	U80771
D-3	225	EST clone (not characterized)	204/204 (100%)	404	U80771
D-4	321	EST clone (not characterized)	319/323 (98%)	587	AK001247
D-5	287	Initiation factor 4 gamma	284/287 (98%)	521	AJ001046
D-6	229	OS-9 precursor	169/170 (99%)	321	U41635
D-7	367	P1-Cdc21	333/339 (98%)	605	X74794
D-8	371	Genomic clone(not characterized)	185/215 (86%)	188	AC004213
D-9	315	Ubiquitin-specific protease UBP41	211/226 (93%)	315	AF079564
D-10	356	Ribosomal protein L3	249/256 (97%)	424	X73460
D-11	257	Peptidyl-prolyl cis-trans isomerase	216/227 (95%)	365	AL049824
D-12	381	Familial Alzheimer disease (presenilin2) gene	370/375 (98%)	664	U50871

color image (Fig. 2 (A)). In the scanning images, red spots correspond to genes more highly expressed in untreated HeLa cells than taxol-treated cells, while, green spots correspond to genes more higyly expressed in taxol-treated cells than in normal untreated cells. Yellow spots represent genes that show no differential expression. Next, we performed a comparison between taxol tolerant HeLa cells and normal HeLa cells. According to the above procedure, mRNA of each cell was extracted. This time, mRNA derived from normal cells was labeled with Cy3 and that from the taxol tolerant cells was labeled with Cy5. The scanning and false color superimposing were carried out in a similar manner (Fig. 2 (B)). The expression ratios of each spot in Fig. 2 are represented as gene pies (Fig. 3). The position of each pie corresponds to the coordinate position of each spot in Fig. 2. The red/green ratios in each respective pie show the Cy3/Cy5 ratios. Fig. 3 (A) and (B) correspond with (A) and (B) in Fig. 2. The DNAs spotted onto the slide glass microarray are the cDNA fragments obtained from HeLa cells as the taxol-inducible genes by DD-RT-PCR. However, DNA microarray analysis between taxol-treated HeLa cells and normal HeLa cells show that no gene was specifically expressed by taxol treatment (Fig. 3 (A)). Although the cause of this result is uncertain, the taxol treatment condition might be too severe for treated cells to be viable enough to recover

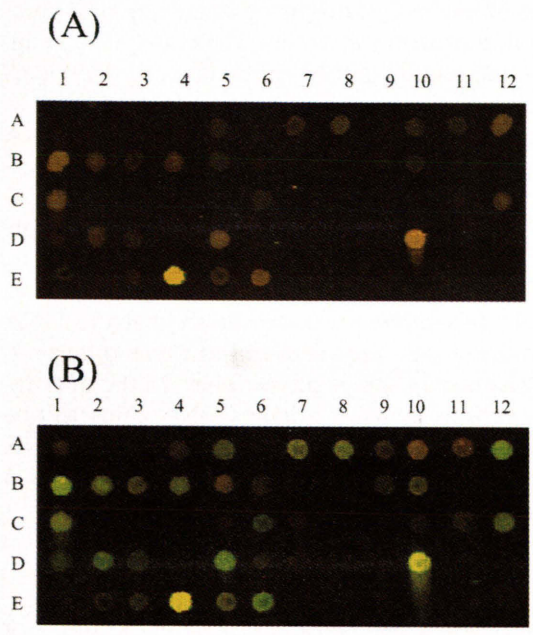

Fig. 2. Expression analysis of the genes differential displayed by DNA microarray.
Two-color image of Hela cell cDNA micro-array. The micro-arrays consist of the 48 PCR-amplified cDNA clones described in Table 1 (A1-D12) The spots on the bottom line are the following control: E1 (lDNA 300bp), E2 (lDNA 1000bp), E3 (pUC18), E4 (β-actin), E5 (hypoxanthin guanine phosphoribosyl transferase), E6 (transferin receptor). (A) A sample mixture of Cy3-labelled first-strand cDNAs generated from Hela cell mRNA (for control) and Cy5-labeled one from taxol treated (0.1 ppm, 6h) Hela cell mRNA was hybridized. (B) A sample mixture of Cy3-labelled first-strand cDNAs generated from taxol-tolerant HeLa cell mRNA and Cy5-labeled one from Hela cell mRNA (for control) was hybridized. After scanning for each fluorescent dye separately, false color images (red for Cy3, green for Cy5) were superimposed.

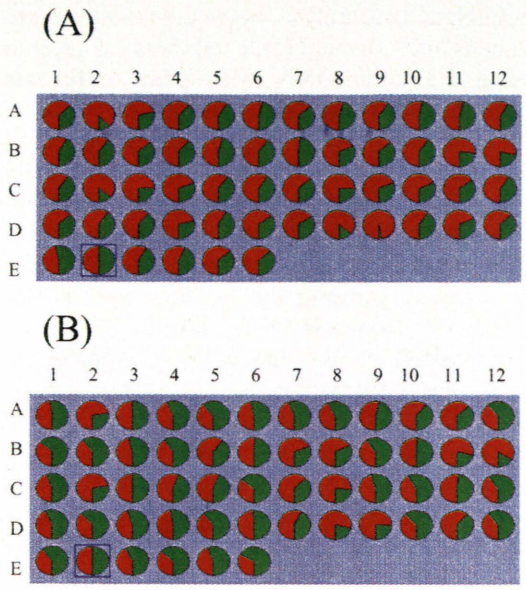

Fig. 3. Gene pie chart representing the percentage of the actual value in light fluorescence units of each probe in the micro-array analysis. The setouts of each pie correspond to that of overlay images shown in Figure 2. (A) A sample mixture of Cy3 labeled first-strand cDNAs generated from HeLa cell mRNA (for control) and Cy5 labeled from taxol treated (0.1 ppm, 6 hr) HeLa cell mRNA was hybridized. (B) A sample mixture of Cy3 labelled one from taxol-tolerating Hela cell mRNA and Cy5 labeled from HeLa cell mRNA (for control) was hybridized. In each pie, the red portion indicates the fluorescence units fraction of Cy3 probe and the green portion indicates the of Cy5 probe based on the scanning data. Blue squares indicate the scanning data of E2 (lambda DNA) were used for a baseline normalizing control for both analysis (A) and (B).

mRNA. Next, an analysis between taxol-tolerant HeLa cells and normal HeLa cells was carried out by using the same DNA microarray to reveale some genes were specifically expressed in taxol-tolerant cells (Fig. 2 (B)). We found several clones specifically expressed in taxol-tolerant HeLa cells, however unfortunately signal strengths was generally weak, so that spots with less than four times strength difference were considered to be false positive clones. Among the genes shown in Table I, only five clones, B-11, B-12, C-8, D-8 and D-9, were positive clones that were specifically expressed in taxol-tolerant cells (Fig. 3 (B)). Among theese, clone D-8 has no homology to reported genes; the other four cDNAs however showed homologies strongly to the human genes. B11 and B12 are both homologous to human caldesmon gene, which is a calcium binding protein distributed in smooth muscle and non-muscle cells, and should plays a vital role in the actomyosin system (Yano *et al.,* 1995). The level of caldesmon (both protein and mRNA) is enhanced by glucocorticoids (Castellino *et al.,* 1995). Recently, it has been demonstrated that glucocorticoids selectively inhibited taxol-induced apoptosis without affecting the ability of taxol to induce microtubule bundling and mitotic arrest (Huang *et al.,* 2000). This finding suggests that apoptotic cell death induced by taxol may occur via a pathway independent of mitotic arrest. It is suggested that glucocorticoids could antagonize taxol-mediated NF-kappaB nuclear translocation and activation through induction of IkappaB-alpha protein synthesis. According to our present study, the transcriptional level of the caldesmon gene was suggested to be elevated by taxol treatment. Concurrently, the endogenous glucocorticoid level should also be raised. Accordingly, antagonism to the NF-kappaB nuclear translocation by the elevation of endogenous glucocorticoid levels should be participate with a taxol-tolerant mechanism. C-8 is a homologue to the putative human Zinc finger transcriptional factor (ZNF207), which was identified as the gene specifically expressed in human vascular smooth muscle cells (VSMC) by differential display RT-PCR (Pahl *et al.,* 1998). The role of ZNF207 has not been clear, this result suggests that ZNF207 participates in the taxol tolerance mechanism. ZNF207 may have a critical role in basic cellular function. D-9 is a homologue of the gene for a ubiquitin-specific protease, UBP41, with isopeptidase activity in chick skeletal muscle (Baek *et al.,* 1997). UBP41 is assumed to play an important role in the recycling of ubiquitin by hydrolysis of branched poly-ubiquitinated protein substrates. The above result suggests that recycling of ubiquitin in a cell might participate in the expression of taxol-tolerance.

In this study, expression analysis of 48 cDNA fragments obtained by DD-RT-PCR was performed by the DNA micro array method. DD-RT-PCR method has been considered to be simple and easy method for differential gene screening, however its reproducibility is believed to be very low. In this study, we have shown that the DNA microarray technique is useful to check differentially displayed false positive clones. In the near future, various kinds of humans DNA chips will be available. However the applicable range of DNA microarray method should be carefully examined because it is a just developing technology.

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