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Short Communication

Identification of gene clusters differentially expressed during the cellular injury responses (CIR) to cisplatin

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Summary The goal of this study was to identify changes in mRNA levels in tumour cells after a toxic exposure to cisplatin (IC_{99} dose). Using suppression-subtractive hybridization (SSH) 2 cDNA libraries were created, an UP library (202 cDNA fragments) and a DOWN library (153 cDNA fragments). Using reversed Northern hybridization 16 and 30 fragments were truly differentially expressed in the UP and DOWN libraries, respectively. Most prominent in the UP library were the mitochondrial and injury response clusters and in the DOWN library the cytoskeletal, protein synthesis and signalling clusters. These distinct clusters potentially represent an expression profile of the cisplatin-induced cellular injury response. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: cisplatin; suppression subtractive hybridization; high throughput screening, gene expression

Cisplatin (cDDP) is a commonly used anticancer agent for treatment of a variety of different malignancies. It is thought to act by forming adducts with cellular DNA, which leads to inhibition of DNA replication (Pinto and Lippard, 1985) RNA transcription (Corda et al, 1991), as well as activation of the cellular injury response (CIR) (Howell et al, 1996). The latter requires a system for detection of DNA damage (Toney et al, 1989), assessment of the extent of the damage (Eastman, 1990) and finally a system to initiate either repair or apoptosis (Eastman, 1990). Each phase in this process from damage recognition to repair or apoptosis needs the participation of a large number of gene products. Therefore, it seems reasonable to assume that the cDDP-induced CIR can be characterized by the sum of changes in expression level of many specific genes (Howell et al, 1996). In view of the fact that little is known about the specific pathways activated by cDDP-induced CIR, the main goal of this study is to identify genes whose expression level is altered after exposure to cDDP and to classify these genes according to their function. For this purpose we combined the suppression subtractive hybridization technique (Diatchenko et al, 1996) with a custom array technology, which has been recently reported to be productive in identifying differentially expressed genes in other model systems as well (Kuang et al, 1998; Johnsson et al, 2000).

MATERIAL AND METHODS

Cells

UMSCC10b human squamous cell carcinoma cells (Grenman et al, 1991) were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM L-glutamine, 100 units ml^{-1} of penicillin G, 100 mg ml^{-1} of streptomycin sulfate and 10% fetal bovine serum (Gibco BRL, Grand Island, NY).

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mRNA extraction

The mRNA used for library construction was isolated from 80% confluent cells by acid guanidium phenol-chloroform extraction followed by isolation of poly(A)+ mRNA using the Oligotex mRNA Midi Kit (Qiagen Inc, Chatsworth, CA). The mRNA used to make cDNA-probes directly from tumour cells was isolated by using the mRNA Direct Kit (Qiagen).

Suppression subtractive hybridization

SSH was performed using the ClonTech PCR-select cDNA Subtraction kit (Clontech Laboratories Inc, Palo Alto, CA) according to the manufacturer's instructions and as described in detail by Johnsson et al (2000).

Preparation of membrane arrays

PCR amplified cDNA fragment from the bacterial clones were spotted onto Magna Graph nylon membranes (Micron Separation Inc, Westborough, MA). Each membrane consisted of a maximum of 108 spots. Water, β-actin, adaptor sequences corresponding to the nested primers and serial dilutions of the whole population of cDNA fragments recovered from forward or reverse SSH steps were included as internal controls (Johnsson et al, 2000).

Membrane hybridizations

Two types of hybridization probes were used in this study. The first was a PCR-amplified probe, containing cDNA fragments recovered from either the forward or reversed SSH step and which putatively contained only cDNA fragments corresponding to differentially expressed mRNAs (Diatchenko et al, 1996; Johnsson et al, 2000). The second probe was used to perform reversed Northern hybridizations. It consisted of cDNA from cDDP-treated and -untreated UMSCC10b cells, prepared by reverse transcription of total cellular mRNA. All probes were labelled with ^{32}P by utilizing the Multiprime Labeling Kit (Amersham Life Science, Arlington Heights, IL), with 20 ng of cDNA per probe (specific

activity ranged from 5×10^7 to 8×10^8 cpm μg^{-1} DNA). Using these probes (5 ng ml^{-1}) membranes were hybridized for 16 h at 68˚C. Hybridizations with subtracted SSH-derived PCR-amplified probes (screening) were performed in triplicate and hybridization with cDNA non-amplified cDNA probes (reversed northerns) were performed in duplicate, triplicate or quadruplet. Analysis of the hybridization was performed with an imaging system from Bio-Rad Laboratories, Hercules, CA and the data were analysed with the PC-based Molecular Analyst Software.

Sequencing and identification of identified fragments

Plasmids containing cDNA fragments that were differentially expressed were sequenced using either primers homologous to the M13 reversed priming site of the plasmid, or nested primers targeted to adaptors 1 or 2R. The sequencing was performed with a 373 XL Automated DNA Sequencer (Perkin-Elmer/Applied Biosystems, Norwalk, CT) at the UCSD Core Facility.

RESULTS AND DISCUSSION

Library construction and differential screening

SSH was used to create a population of cDNA fragments corresponding to mRNAs whose levels were either increased (the UP library) or decreased (the DOWN library, created by the reversed subtraction using the non-treated cells as tester) in the UMSCC10b cells exposed to an IC_{99} concentration (50 μM) of cDDP relative to the untreated UMSCC10b cells. Figure 1 presents a flow diagram of the yield from each step of the isolation procedure.

The PCR products generated from the bacterial inserts were screened for differential expression with PCR-amplified SSH probes on membrane arrays (Diatchenko et al, 1996; Johnsson et al, 2000). Array elements demonstrating >5-fold differences in abundance in the UP and DOWN subtracted libaries in at least 1 of 3 repeat hybridizations were selected for further investigation. Based on this criterion, 26 (13%) and 44 (29%) fragments were identified as being differentially expressed in the UP and DOWN library, respectively. Although modest, these percentages are of the same order of magnitude as demonstrated in a recent study from this laboratory (Johnsson et al, 2000).

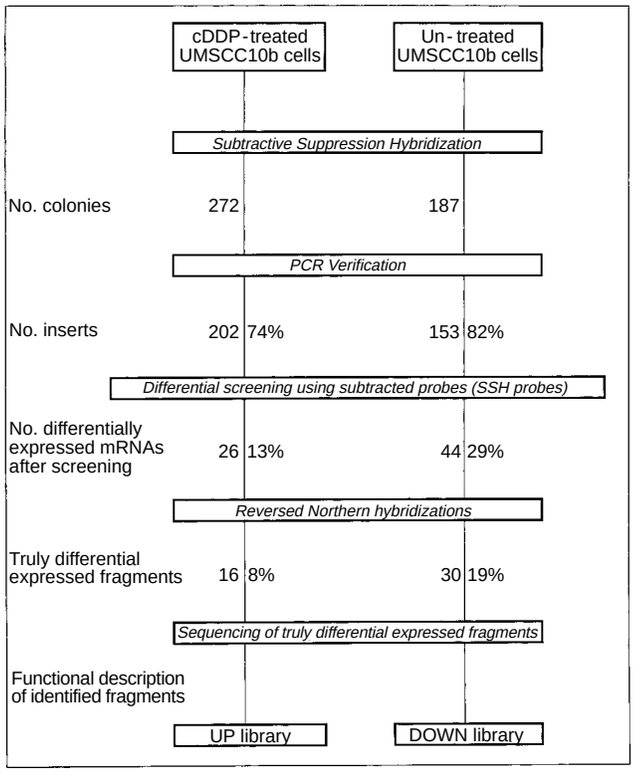

Figure 1 Flow diagram showing the number of cDNA fragments processed for the UP and DOWN libraries

Magnitude of differential expression determined by reversed Northern blot analysis

The magnitude of the difference in abundance between the mRNA levels in the cDDP-exposed and non-exposed UMSCC10b cells were examined further by reversed Northern blotting. 70 cDNAs present in the UP (26) and DOWN (44) libraries were spotted onto membrane arrays and hybridized with cDNA probes prepared from mRNA of cells exposed and non-exposed to cDDP (IC_{99}). Hybridizations were repeated 2 to 4 times and the mean level of differential expression for each element in the array was calculated as the ratio between cDDP-exposed and -non-exposed cells. Of the 26 cDNAs present in the UP library, 16 (62%) corresponded to mRNAs that demonstrated at least a 1.6-fold difference in level. Among these 16 cDNAs 4 showed at least a 4-fold difference and 1 at least a 10-fold difference (Table 1). Of the 44 cDNAs in the DOWN library, 30 (68%) demonstrated at least a 1.6-fold difference in expression. Among these 6 cDNAs showed at least a 4-fold difference. None had a 10-fold difference (Table 2). The finding that the majority of mRNAs show little change, and a progressively smaller fraction shows incrementally larger changes is consistent with results obtained in other systems where either isogenic cells growing under different conditions have been compared (Zhang et al, 1997) or in a steady state condition where tumour cells resistant to cDDP were compared to their sensitive variant (Johnsson et al, 2000).

Validation of the magnitude of expressing was obtained using 1 or 2 fragments in each library present multiple times. In the DOWN library β-actin and α-tubulin were identified 4 and 3 times, respectively. The mean expression of their expression ratios was 3.4 ± 1.5 and 2.9 ± 0.9, respectively. In the UP library MDM2 was present in 4 different bacterial clones, resulting in expression levels 1.9 ± 0.1. Overall, the mean variance is small and confirms previous observations of biological variation between samples (Zhang et al, 1997; Johnsson et al, 2000).

Identification of cDNA fragments

The cDNA fragments corresponding to the 16 mRNAs in the UP library and 30 mRNAs in the DOWN library that demonstrated at least a 1.6-fold increase or decrease in expression were sequenced. 43 (92%) of these were identifiable as segments of cDNAs contained in GenBank, 1 (2%) was an EST and 3 (6%) were unknown. Some genes were identified more than once. mRNA encoding mitochondrial genes and MDM2 were identified multiple times in the UP library, and β-actin, and α-tubulin were found more than once in the DOWN library. In the UP library 2 clusters of genes can be identified (Table 1). The first cluster consists of mitochondrial related genes, the 16S rRNAs and BC200. The human mtDNA encoding rRNAs (rRNAs 12 S and 16S rRNA) are involved in the synthesis of several subunits of

Table 1 Truly differentially expressed fragments present in the UP library

Clone number	Fragment identity	GenBank identity	GenBank accession #	Fold increase in expression	Function
326	mitochondrion (2851–3089)	HUMMTA	*D38112*	13.7	***Ribosomal products***
230b	mitochondrion (2849–3124)	HUMMTA	*D38112*	6.2	
119b	mitochondrion (2332–2544)	HUMMTA	*D38112*	3.9	mtDNA coding for 16S rRNA
313	mitochondrion (2282–2629)	HUMMTA	*D38112*	2.1	
46a	BC200	HSBC200RNA	*U01305*	2.1	Associated with 5S rRNA
					Genes involved in the CIR
83b	hMSH6	HSU54777	*U54777*	3.5	DNA damage recognition and repair
203b	HSP90	HSHSP90R	*X15183*	1.8	Early response gene, heat shock protein
339	MDM2	F144014S16	*AF144029*	2.0	
336	MDM2	F144014S16	*AF144029*	1.9	
280	MDM2	F144014S16	*AF144029*	1.9	Negative feedback for p53
247	MDM2	F144014S16	*AF144029*	1.7	
					Function unknown
165b	DDX1 (dead box protein)	HSCL1042	*X70649*	2.4	Translation, RNA splicing and RNA stability
299	NICE-5 protein	NM_017582	*NM_017582*	1.8	Part of human epidermal differentiation complex
65b	Human clone JkA7 mRNA	HSU38436	*U38436*	1.7	unknown
94b	KIAA0017	HUMRSC399	*D13642*	4.1	unknown
242	unknown			1.6	unknown

among others the cytochrome C oxidase complex, ATPases and the NADH reductase complex (Darnell et al, 1990), all known to be potentially associated with the apoptotic process. Little is known about the function of BC200, however, it has been shown that BC200 is associated with polymerase III transcripts, which by itself makes a variety of small stable RNAs including the small 5S RNA of the ribosome (Kremerskothen et al, 1998).

A second cluster consists of genes directly involved in the cellular injury response (CIR) such as hMSH6, HSP90 and MDM2. The hMSH6 is a part of the DNA mismatch repair system and forms a complex with hMSH2 that recognizes and initiates repair of DNA damage (Lage et al, 1999). In analogy of its role in DNA mismatch repair, it has been shown that hMSH6 may also play a role in the recognition of cDDP-DNA adducts (Fink et al, 1996) and therefore may play a role in the initiation of the CIR. Another important player in the CIR is HSP90 (Itoh and Tashima, 1991). HSP90 gets induced by cDDP under conditions of severe damage as was demonstrated in mice kidney cells with cDDP-induced acute renal failure and degenerative changes in epithelial cells (Satoh et al, 1994). Part of the function of HSP90 is to chaperone the folding of proteins and by doing so play a role in regulating signal transduction pathways that control cell growth and survival. A third member of this CIR cluster is the *MDM2* gene, an important player in the p53 pathway through the formation of a MDM2–p53 auto-regulatory feedback loop (Oliner et al, 1993). For several other identified cDNA fragments the role in the CIR is less clear. The dead box protein gene *DDX1* might be involved in translation, RNA splicing and RNA stability, while the function of NICE-5, JkA7, KIAA0017 and cDNA fragment are unknown.

In the DOWN library 3 functional gene clusters could be identified, involving cell structure, signalling and protein translation. The first cluster contains genes such as α-tubulin, β-actin, cytokeratin, actin-binding protein, keratin-related protein, cytokeratin 13, and thymosin β4. The changes in expression level of a number of cytoskeletal components suggest a reorganization of the microfilament system in response to the cDDP-induced injury which has also been observed during the process of cell death (Brancolini et al, 1997). Cells retract from adhesion substrate, sever the contacts with neighbouring cells, and actin filaments will be concentrated in the perinuclear area in a ring-like organization, resulting in among others, the down-regulation of the actin-binding protein and thymosin β_4, an actin-sequestering protein (Huff et al, 1999) and the rupture of α-tubulin during the apoptotic process (Kato, 1999). The function of the Wilm's tumour-related protein in human is unknown, however, its yeast homologue GRC5, seems to be an essential yeast gene for the establishment of a proper cytoskeletal structure (Koller et al, 1996). Summarizing, the parallel down-regulation of the expression level of so many cytoskeletal-related genes strongly suggests a well-orchestrated process dismantling the structure of the cell.

A second cluster contains a set of genes involved in various signalling pathways. Transketolase and the thyroid hormone-binding protein are both part of the metabolic energy pathways in the cell. Transkelotase is part of the pentose phosphate pathway (Pontremoli and Grazi 1968) and the thyroid hormone-binding protein gene encodes a monomer of pyruvate kinase, which can lead to decreased pyruvate kinase activity (Dieudonne et al, 1999). Two other genes, *ferritin H chain* and transcription factor *HSF4b*, are involved in pathways activated by oxidative stress or damage. Ferritin synthesis plays a major role in the prevention of cellular damage (Balla et al, 1992) while the transcription factor *HSF4b* acts as a transcriptional activator of the heat-shock proteins (Tanabe et al, 1999). This cluster also contains the interferon-inducible gene family *1-8U*. This gene is induced by both type 1 (α and β) and type II (γ) IFN. The function of IFN-inducible gene *1-8U* remains, however, unclear. Taken this all together, it is clear that the down-regulation of genes present in this cluster negatively affects the cells' energy metabolism and the ability to react on stress.

A third cluster in the DOWN library indicates that the protein synthesis is reduced at several levels. First the expression levels of elongation factors 1-alpha and 1-gamma, which are known to

Table 2 Truly differentially expressed fragments present in the DOWN library

Clone number	Fragment identity	GenBank identity	GenBank accession #	Fold increase in expression	Function
					Signaling
119	PABPCI	HUMPOLYAB	*NM 002568*	6.7	mRNA stability
159	transcription factor HSF4b	AB029348	*AB029348*	2.3	transcriptional activator of HSP
44	Ferritin H chain	HUMMFERH	*M11146*	2.3	cellular damage prevention
196	Interferon-inducible gene	HS18U	*X57352*	1.6	part of the IFN pathway
126	Transketolase	HSU55017	*U55017*	2.3	pentose phosphate pathway
38	Thyroid hormone binding protein	HUMTCBA	*M26252*	8.7	affect pyruvate kinase activity
					Protein Synthesis
66	Elongation factor 1-α	HUMEF1A	*J04617*	6.9	protein translation
127	Elongation factor 1-γ	HSEF1GMR	*Z11531*	2.0	protein translation
116	Acidic ribosomal phosphoprotein	HUMPPARPO	*M17885*	2.6	ribosome regulation
25	Ribosomal protein S29	HSU14973	*U14973*	2.5	ribosome regulation
195	Ribosomal protein L41	HSRPL41	*Z12962*	1.7	ribosome regulation
199	Proteasome subunit HC3	HUMPSC3	*D00760*	2.0	protein breakdown
					Cytoskeletal Organization
100	β-actin	HSAC07	*X00351*	5.6	cytosketal organization
109	β-actin	HSAC07	*X00351*	2.8	protein synthesis
53	β-actin	HSAC07	*X00351*	2.6	cytoskeleton dynamics
192	β-actin	HSAC07	*X00351*	2.5	differentiation
35	Actin-binding protein	HSABP280	*X53416*	2.7	
9	Keratin-related protein	HSKERELP	*X62571*	4.4	
154	α-tubulin	HUMTUBAK	*K00558*	4.0	
139	α-tubulin	HUMTUBAK	*K00558*	2.7	
189	α-tubulin	HUMTUBAK	*K00558*	2.1	
153	Wilm's tumor related protein	HUMQM	*M64241*	2.5	
6	Cytokeratin 13	HSCYTK	*X52426*	3.8	
15	Thymosin β_4	HUMTHYB4	*M17733*	3.5	actin sequestering protein
					Function unknown
22	Cloride ion current inducer	HSU53454	*U53454*	2.7	unknown
47	Insulinoma gene	HUMIDB	*J02984*	2.5	possible DNA-binding protein
122	Retinal pigment epithelium	HUMRETP1GB	*L07393*	2.5	unknown
36	Unknown		*NM_003917*	2.4	unknown
106	cDNA FLJ11896 fis	AK021958	*AK021958*	2.3	unknown
81	Unknown			1.6	unknown

mediate the binding of aminoacyl-tRNAs to acceptor sites of ribosomes during protein synthesis (Duttaroy et al, 1998), are down-regulated. Second, exposure to cDDP negatively affects the synthesis as well as the number of active ribosomes as demonstrated by the decrease in level of expression of several ribosomal RNAs (Jordan and Carmo-Fonseca, 1998). Thirdly, the expression level of the proteasome subunit *HC3*, a gene involved in protein breakdown is reduced as well and may serve as a feedback reaction on the reduced protein production (Tiao et al, 1997). For the remaining genes the function is either unknown or not well understood yet.

In summary, the present study used a PCR-based subtraction strategy to provide an analysis of the cellular injury response to cDDP. The cytotoxic response to cDDP in UMSCC10b cells involved the induction as well as the repression of genes. The majority of these genes identified as differentially expressed could be grouped in clusters based on their functional description. These clusters included genes involved in energy production, response to damage, organization of the cytoskeleton, protein synthesis and signalling transduction, providing a map for an expression profile associated with the injury response to cDDP, underlining the feasibility of using a molecular profile or fingerprint of anticancer drugs in tumours as a prognostic tool.

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