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 (IC₉₀ 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

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

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).

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 ³²P by utilizing the Multiprime Labeling Kit (Amersham Life Science, Arlington Heights, IL), with 20 ng of cDNA per probe (specific

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activity ranged from $5 \times 10^7$ to $8 \times 10^8$ 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 libraries 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).

**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 was 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 $3.4 \pm 1.5$ and $2.9 \pm 0.9$, respectively. In the UP library MDM2 was present in 4 different bacterial clones, resulting in expression levels $1.9 \pm 0.1$. 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
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 II 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 regulation 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, 1996) and therefore may play a role in the initiation of the CIR. 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. Transketolase 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 (Ball 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 all this 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

| 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  |
| 313          | mitochondrion (2282–2629) | HUMMTA | D38112               | 2.1  |
| 46a          | BC200            | HSBC200RNA      | U01305               | 2.1  | Associated with 5S rRNA |
| 83b          | hMSH6            | HSU54777        | U54777               | 3.5  | Genes involved in the CIR |
| 203b         | HSP90            | HSHSP90R        | X15183               | 1.8  | DNA damage recognition and repair |
| 339          | MDM2             | F144014S16      | AF144029             | 2.0  | Early response gene, heat shock protein |
| 336          | MDM2             | F144014S16      | AF144029             | 1.9  |
| 280          | MDM2             | F144014S16      | AF144029             | 1.9  |
| 247          | MDM2             | F144014S16      | AF144029             | 1.7  |
| 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  |
| 94b          | KIAA0017         | HUMRSC399       | D13642               | 4.1  |
| 242          | unknown          | unknown         | unknown              | 1.6  |

Table 1. Truly differentially expressed fragments present in the UP library.
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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