Identification of the genes responsive to etoposide-induced apoptosis: application of DNA chip technology

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Abstract DNA chip technology was used in an attempt to identify target genes responsible for apoptosis induced by etoposide, a p53 activating topoisomerase II inhibitor used clinically as an antitumor agent. 62 Individual mRNAs whose mass changed significantly were identified after screening oligonucleotide arrays capable of detecting 6591 unique human mRNA species. 12 (Nine induced and three repressed) of the etoposide-responsive genes were further studied by Northern analysis and an agreement rate of 92% was reached. Among the 12 genes studied, two (WAF1/p21 and PCNA) are known p53 regulatory genes, two (glutathione peroxidase and S100A2 calcium-binding protein) appear to be the novel p53 target genes and the others appear to be p53-independent. Based upon these findings, the signalling pathways that possibly mediate etoposide-induced apoptosis are proposed.

Key words: Apoptosis; DNA chip; Etoposide; Gene expression; p53

1. Introduction

Etoposide (VP16) is a DNA topoisomerase II inhibitor [1]. It has been shown to be active against a variety of tumor cells in both preclinical and clinical studies [2]. Etoposide is used as an antitumor agent in clinic, mainly for the treatment of testicular cancers and small cell lung carcinomas [2,3]. The main toxicity derived from the use of etoposide is bone marrow depression with leucopenia and thrombocytopenia. Other side effects include nausea, diarrhea, mucositis, and hypotension [2,3]. As a DNA damaging reagent, etoposide has been shown to induce apoptosis in a variety of tumor cell lines harboring either wild-type or mutant p53 [4,5]. Although the entire signalling pathways mediating etoposide-induced apoptosis are not clear, one pathway could involve p53, since DNA damage induced by etoposide has been shown to activate the p53 activity [6].

In an attempt to identify the genes responsive to or responsible for etoposide-induced apoptosis and to reveal the apoptosis signalling pathways triggered by a DNA damaging reagent, we have utilized the DNA chip technology to simultaneously display changes of gene expression. A set of four oligonucleotide arrays, representing 6591 known genes, was screened. Among the 12 genes characterized, some are previously known as p53-responsive genes, while others are the novel p53 target genes and still others are involved in p53-independent signalling pathways. Characterization of these etoposide-responsive genes may lead to the identification of novel target(s) for the discovery of anticancer drugs.

2. Materials and methods

2.1. Cell cultures

U2-OS cells, a human osteogenic sarcoma cell line with endogenous wild-type p53, were grown in 10% McCoy's 5A medium. For drug treatment, subconfluent cells were incubated with etoposide (25 or 50 μM, Sigma) for 6 or 24 h. The control cells were treated with DMSO for 24 h.

2.2. cDNA probe preparations

cDNAs encoding Waf-1 [7], GPX (a gift from Dr. Larry Oberley at the University of Iowa) were used as probes. The following probes were generated by RT-PCR as described [8] using human placenta RNA (Oncor) as the template: calcyclin/S100A6, proliferating cell nuclear antigen (PCNA), ornithine carboxylase (ODC), heterogeneous nuclear ribonucleoprotein core protein A1 (HnRNP), metallothionein-II (MT-2), S100 calcium-binding protein A2 (S100A2), transforming growth factor-β type II receptor (TGFβ-RII) and thioredoxin (TXN). The primers were designed based upon the published sequence (see Table 1), flanking the entire open reading frame. A single PCR fragment with expected size was gel-purified. Some of them were subcloned and sequenced. Tubulin β-1 chain (accession #: T65580) and ferritin light chain (accession #: T72863) were obtained from the Washington University/Merck EST bank. The clones were PCR amplified and sequenced. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was obtained from Ambion.

2.3. Northern analysis

Total RNA was isolated from etoposide-treated cells using RNAzol solution (Tel-Test). 15 μg of total RNA or 1.8 μg of poly (A+) RNA was subjected to Northern analysis as previously described [9]. Densitometric quantitation was performed on a densitometer (Molecular Dynamics) or on a phosphoimager (STORM 860 Molecular Dynamics).

2.4. DNA chip technology

Affymetrix oligonucleotide arrays were used for mRNA expression profiling [10,11]. Polyadenylated RNA was isolated from 5×10^7 lysed cells using Qiagen Oligotex. Double stranded cDNA was prepared in 20 μl volumes using Life Technologies Superscript Choice System and an oligo-$(dT)_{24}$ anchored T7 primer [10]. The final reaction conditions for the first strand synthesis were 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μm of each of dATP, dCTP, dGTP, dTTP, 100 pmol primer, 0.1–5 μg of mRNA and 200 U Superscript II reverse transcriptase/μg mRNA, 1 h at 37°C. Second strand synthesis was carried out for 2 h at 16°C in 150 μl volumes containing 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM β-NAD^+, 250 μm of each of dATP, dCTP, dGTP, dTTP, 1.2 mM DTT, 65 U/ml DNA ligase, 250 U/ml DNA

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Abbreviations: GPX, glutathione peroxidase; HnRNP, heterogeneous nuclear ribonucleoprotein core protein A1; MT-2, metallothionein-II; ODC, ornithine carboxylase; PCNA, proliferating cell nuclear antigen; S100A2, S100 calcium-binding protein A2; TGFβ-RII, transforming growth factor-β type II receptor; TXN, thioredoxin

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Table 1
Primers used for the generation of cDNA probes by RT-PCR

Genes	Primers	Expected size	References
Calcyclin/S100A6	Cal-01, 5′-ATGGCATGCCCCCTGGATC-3′ Cal-02, 5′-TCAGCCCTTGAGGGCTTCAT-3′	270 bp	Accession # J02763
HnRNP	HnRNP-01A, 5′-GCCGTCATGTCTAAGTCAGA-3′ HnRNP-02, 5′-TTAAAATCTTCTGCCACTGCC-3	960 bp	Accession # X12671
MT-2	MT-01, 5′-GGAATTCGCCGCCATGGATCCCAACTGC-3′ MT-02, 5′-GGAATTCCCAGCATCAGGCGCAGC-3′	212 bp	Accession # J00271
ODC	ODC-01, 5′-GGAATTCATGAACAACTTTGGTAAT-3′ ODC-02, 5′-GGAATTCAAGCTAAACTTGCAGTT-3′	1437 bp	[28]
PCNA	PCNA-01, 5′-GGAATTCCCGCCTGCCTGTAG-3′ PCNA-02, 5′-GGAATTCTTGAATTTTAAGAATGCC-3′	855 bp	[29]
S100A2	S100A2-01, 5′-ATGTGCAGTTCTCTGGAGCA-3′ S100A2-02, 5′-TCAGGGTCGGTCTGGGCA-3′	294 bp	Accession # Y07755
TGFβ-RII	TGFR-01, 5′-ATGGGTCGGGGGCTGCTC-3′ TGFR-02, 5′-GCTATTTGGTAGTGTTTAGGG-3′	1.7 kb	Accession # M85079
TXN	TXN-11, 5′-GGAATTCGCCAAGATGGTGAAGCA-3′ TXN-12, 5′-GGAATTCAGAAAACATGATTAGAC-3′	348 bp	[30]

polymerase I, 13 U/ml RNase H followed by 5 min at 16°C with 67 U/ml T4 DNA polymerase. The cDNA synthesis was terminated by the addition of 10 μl of 0.5 M EDTA. Double stranded cDNA products were purified by phenol:chloroform:isoamyl alcohol (25:24:1 saturated with 10 mM Tris-HCl, pH 8.0/1 mM EDTA) extraction, phase separation using Phase Lock Gels (PLG1, 5′-3′) and ethanol precipitation. Biotinylated RNA was synthesized using an Ambion T7 Megascript system with biotin-11-CTP and biotin-16-UTP (7.5 mM ATP, 7.5 mM GTP, 5.625 mM unlabelled UTP, 1.875 mM bio-UTP, 5.625 mM unlabelled CTP, 1.875 mM bio-CTP) for 4 h at 37°C. In vitro transcription products (IVT) were purified using Qiagen RNAeasy columns. Biotinylated RNA products were fragmented for 35 min at 94°C in a buffer composed of 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate. Affymetrix GeneChip arrays (HUM6000) were hybridized with the biotinylated IVT products (10 μg/chip) for 16 h at 40°C. using the manufacturer's hybridization buffer. After washing the arrays, hybridized RNA was detected using streptavidin-phycoerythrin staining (6×SSPE, 0.05% Triton X-100, pH 7.6, 1 mg/ml acetylated bovine serum albumin, 2 μg/ml streptavidin-phycoerythrin from Molecular Probes) for 10 min at 40°C. The DNA chips were scanned using a specially designed confocal scanner made for Affymetrix by Hewlett-Packard (commercially available through Affymetrix, Santa Clara, CA, USA). The excitation source was an argon ion laser and the emission was detected by a photomultiplier tube through a 570 nm longpass filter. Digitized image data were processed using the GeneChip software (version 3.0) from Affymetrix as described previously [10]. RNA abundance was determined based on the average of the differences between perfect match and mismatch intensities for each probe family. Gene induction or repression was considered significant if the change in average difference intensity was above 2-fold [12].

3. Results

3.1. Identification of the genes responsive to apoptosis induced by etoposide: the use of DNA chip technology

We have previously shown that etoposide, a topoisomerase II inhibitor, induces p53 DNA-binding and transactivation in U2-OS, a human osteogenic sarcoma cell line harboring wild-type p53 [6]. We have also observed that after etoposide treatment, cells undergo apoptosis as revealed by morphological changes such as cell shrinkage and detachment and by DNA fragmentation (data not shown). In an attempt to identify genes whose expression changed during etoposide-induced apoptosis, we utilized DNA chip technology to obtain an overall profile of gene expression. Since etoposide induces apoptosis in both p53 positive and p53 negative cells [4,5], both p53-responsive and non-responsive genes are expected to be identified. In order to identify both early and late apoptosis-responsive genes, poly (A+) mRNA was isolated from cells treated with etoposide (25 μM) for 6 h (neither obvious morphological change, nor DNA fragmentation) and for 24 h (obvious morphological signs of apoptosis/DNA fragmentation) and subjected to oligonucleotide chip hybridization. Cells treated with DMSO for 24 h were used as the control. A total of four chips, containing 6591 genes were screened. The hybridization results from all four chips were compiled and sorted on the basis of fold change compared to control cells. Genes that displayed approximately two-fold or greater changes were scored as significant changes [12]. 62 mRNA

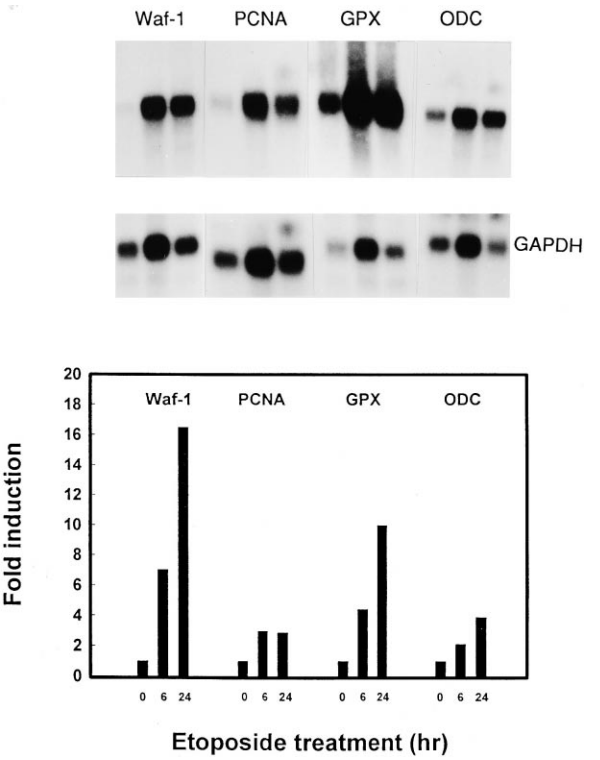

Fig. 1. Induction of Waf-1/p21, PCNA, GPX and ODC by etoposide. Subconfluent U2-OS cells were treated with etoposide (25 μM) for 6 h or 24 h. Poly (A+) mRNA was isolated and either subjected to chip hybridization (Table 2) or Northern analysis (1.8 μg). The top panel is the expression of mRNA encoding Waf-1/p21, PCNA, GPX and ODC, as indicated. The bottom panel is the fold induction of expression after normalization with GAPDH for each individual gene. Quantitation was performed on a densitometer.

Table 2
Comparison of expression changes detected by chips and Northern in U2OS cells treated by etoposide

Gene	DNA Chip			Northern		
	6 h	24 h	Diff Call	6 h	24 h	Diff Call
Calcyclin	ND	2.4	I	1.5	4.3	I
Ferritin LC	0.1	ND	D	0.8	0.1	D
GPX	1.8	2.7	I	5	10	I
hnRNP 1	0.5	0.4	D	0.6	0.7	D
MT-2	2.3	2.7	I	1.1	1.7	I
ODC	ND	1.9	I	3	4	I
PCNA	2.5	ND	I	3	3	I
S100 A2	ND	2.2	I	1.5	3	I
TGFβ-RII	ND	2.3	I	0.8	3.6	I
Tubulinβ_1	ND	0.3	D	0.6	0.5	D
TXN	1.8	ND	I	1	1.3	NC
WAF-1	1.1	2.4	I	7	17	I

Diff Call, Difference Call; I, Increased; D, Decreased; NC, No Change; ND, Not detected.

species out of the 6591 genes were identified by these criteria. Of these 62 mRNA species 41 were significantly induced and 21 were significantly repressed at either or both of the time points (data not shown). Based upon potential involvement in apoptosis signalling pathways, 12 genes (nine induced and three repressed) were selected for further analysis and characterization (see Table 2).

3.2. *Confirmation of etoposide-inducible genes by Northern analysis*

To confirm the results obtained from the chip hybridization, we used the same batch of mRNA and performed Northern analysis in a subset of etoposide-inducible genes. These include Waf-1/p21 and PCNA, two known p53 regulatory genes [13,14], and GPX and ODC, two genes unknown for p53 regulation. GPX is a primary antioxidant enzyme to decompose hydrogen peroxide, thus preventing oxidative stress-induced damage [15], whereas ODC is a rate-limiting enzyme for polyamine biosynthesis, and required for G1 progression [16]. As shown in Fig. 1 (top), all four genes are indeed induced by etoposide at both 6 h and 24 h. The fold induction after normalization with the housekeeping gene, GAPDH, is shown at the bottom of the figure. A greater fold induction is observed using Northern analysis, compared to the chip hybridization (Table 2). This may reflect more stringent hybridization conditions used for chip hybridization. The threshold we set for inclusion as etoposide regulatory genes is, therefore, valid in this subset of genes examined.

We next performed Northern analysis to further confirm the chip results in a larger set of etopside-responsive genes detected by the chip assay. We chose eight additional genes whose expression as detected by chip was either induced (five out of eight) or repressed (three out of eight) by etoposide treatment. As shown in Fig. 2 and Table 2, expression patterns for seven out of eight genes correlate with the chip results. The only disagreement is seen in the gene encoding thioredoxin in which Northern does not show a significant induction. Overall, 20% (12 out of 62) of etoposide-reponsive genes detected by the chip were examined with Northern analysis and one false positive was found (Table 2).

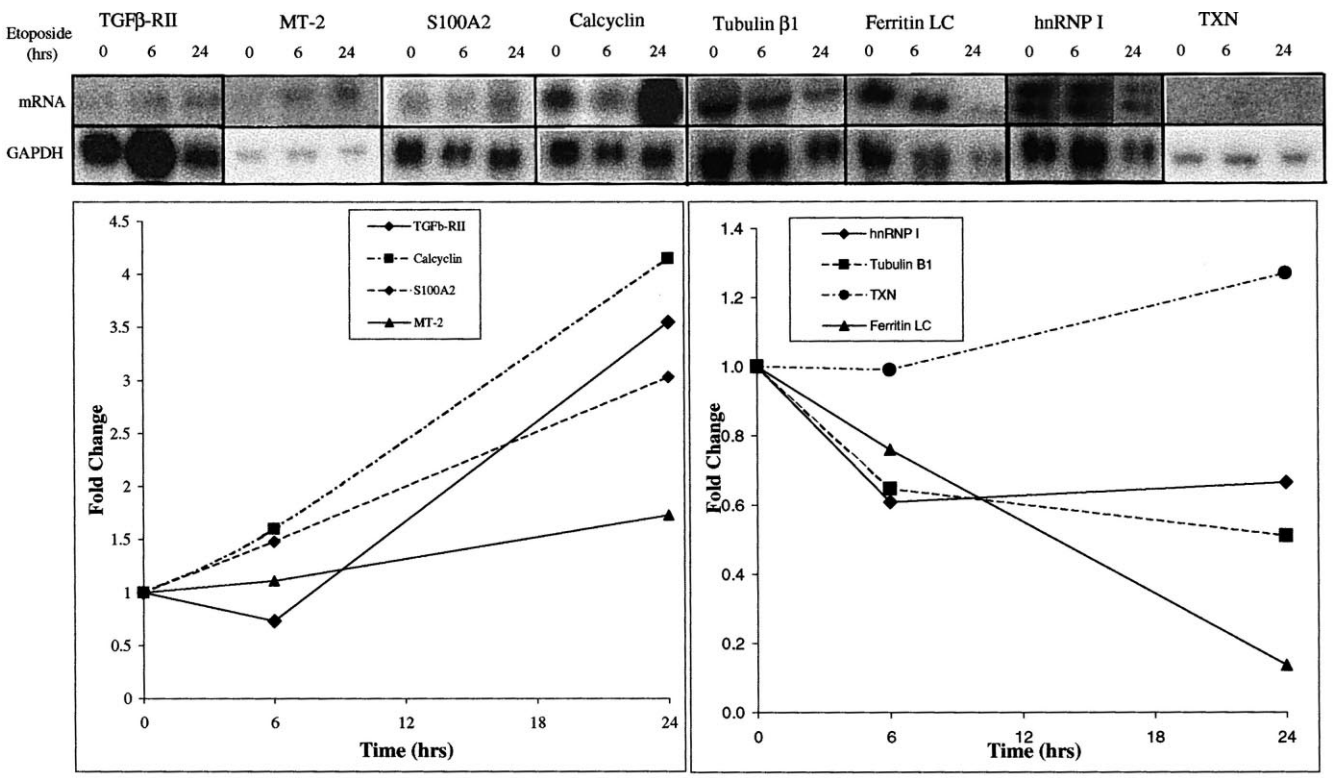

Fig. 2. Validation of the chip results by Northern analysis. Total RNA was isolated from U2-OS cells treated with etoposide (25 μM) for 6 and 24 h and subjected (15 μg) to Northern analysis. Due to the low abundance of TGFβ-RII transcript in U2-OS cells, 1.8 μg poly(A+) RNA (the same batch used for chip hybridization) was loaded for Northern analysis. Northern blots were first hybridized with the indicated cDNA probes and then with GAPDH. The blots were exposed in a phosphoimager, quantitated against GAPDH and expressed as fold changes.

Table 3
Identification of putative p53 binding sites in the etoposide-inducible genes[1]

Gene Name	Accession	p53 binding site sequence	Location
Consensus p53 binding site		RRRCWWGYYYRRRCWWGYYY[2]	Promoter or intron
CDK-inhibitor p21 (WAF1)	U50603	GAACATGTCCAACATGTTG	Promoter
PCNA	J05614	ACATATGCCCGGACTTGTTC	Promoter
Calcyclin/S100A6	J02763	GGGCTTGGCCGAGCTGGCCT	Promoter
Glutathione peroxidase (GPX)	Y00483	GGGCCAGACCAGACATGCCT	Promoter
Ornithine decarboxylase (ODC)	M81740	AAGTTTGTTTGAATTAGCCC	Intron 1
S100 calcium-binding protein A2	Y07755	GGGCATGTGTGGGCACGTTC	Promoter
Thioredoxin	X70286	AGACTTGCCTAGAGTTACCC	Promoter

[1]No space between the two 10 bp motifs and allow two mismatches.
[2]R: A or G, W: A or T, Y: C or T.

3.3. *Identification of potential novel p53-dependent genes up-regulated during etoposide-induced apoptosis*

Among etoposide-inducible genes, we expect to identify both p53-responsive and non-responsive genes. We, therefore, searched for the consensus sequence of the p53 binding site in the promoter or intron regions of all 41 etoposide-inducible genes. We allowed two mismatches to the consensus p53 binding sequence but no spacer between the two 10 bp motifs, since almost all endogenous p53 target genes have a few mismatches but no spacer [17]. Based upon the available promoter/intron sequences deposited in the GenBank, we identified, as listed in Table 3, putative p53 binding sites in the promoter of the genes encoding the following proteins: calcyclin/S100A6 (a growth regulated, calcium-binding protein) [18], GPX, S100A2 (a calcium-binding protein) [19] and TXN, a redox sensitive protein [20], and also in the first intron of the gene encoding ODC. None of the five genes was previously known as a p53 target gene. Through extensive analyses including a gel shift assay (for specific p53-binding), a transactivation assay (for p53-dependent target gene activation) and Northern analysis (for p53-dependent induction of endogenous target genes by etoposide), we concluded that GPX and S100A2 are novel p53 target genes [21,22].

3.4. *Comparison of p53 regulatory genes identified by both chip and series analysis of gene expression (SAGE) technologies*

SAGE technology which can be used to identify both known and unknown genes [23] has been recently utilized to identify genes responsible for p53-induced apoptosis [24]. A total of 65 p53-responsive genes (32 were induced and 33 were repressed) was identified after sequencing 101 694 tags, representing 7202 unique transcripts [24]. 32 Out of these 65 genes, but none of 13 p53-induced genes (*PIG*s) [24] were found among the 6591 genes on the DNA chips. To examine whether two techniques can identify the same set of the responsive genes, we compared the 32 p53-responsive genes by SAGE and 62 etoposide-responsive genes by chip hybridization. Although apoptosis inducers (etoposide versus adenovirus-p53) and cell lines (U2-OS versus DLD-1) used in the two experiments were different, we are able to find six (three inducible and three repressed) identical genes picked up by both

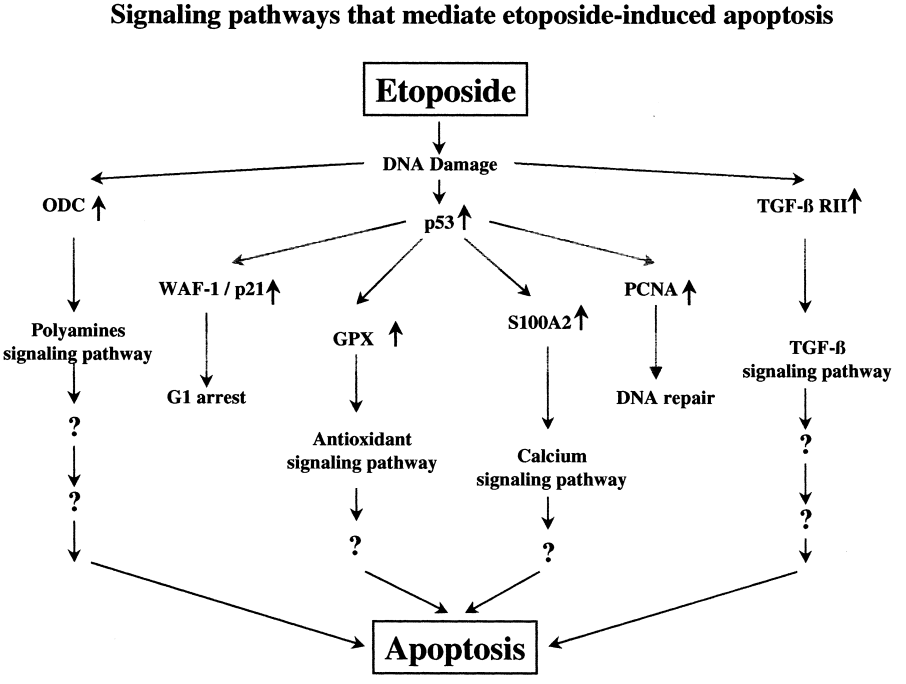

Fig. 3. Signalling pathways that mediate etoposide-induced apoptosis. DNA damage induced by etoposide activates p53 and triggers the p53 signalling pathway which in turn activates the antioxidant pathway as well as the calcium signalling pathway. On the other hand, DNA damage also triggers the polyamine pathway by inducing ODC expression and the TGFβ signalling pathway by inducing expression of the TGFβ type II receptor. All these signal transduction pathways converge and apoptosis results.

Table 4
The p53-regulated genes identified by both chip and SAGE technologies

Genes induced	Genes repressed
CDK inhibitor p21 (WAF1)	Beta-tubulin
40S ribosomal protein	hnRNP core protein
Human mRNA (clone 9112)	90 kDa heat-shock protein

technologies (Table 4). This indicates that the two technologies with different methodological approaches, can identify the same set of genes subjected to p53 regulation.

4. Discussion

4.1. Application of DNA chip technology in the detection of etoposide-responsive genes

To understand the signal transduction pathways leading to apoptosis induced by etoposide, an anticancer drug used in clinic, we utilized DNA chip technology to determine the changes of the gene expression profile. Exposure of cells to etoposide initiates a complex set of cellular responses as reflected by a global change of gene expression. Among the 6591 genes screened, we have identified 62 genes (either being induced or repressed) responsive to etoposide-induced apoptosis. Some changes may confer the cellular defense against DNA damage, while others may potentiate the damage when repair becomes impossible. Among these 62 genes, we characterized 12 genes by Northern analysis. Although the fold changes were different between the two methods, which varied by a number of factors including stringency of hybridization, variations in sample preparations, different ways in normalization for sample loading and so on, an agreement of 92% was reached. This provides the assurance that the DNA chip technology can be used as a rapid tool with a high accuracy to examine global changes of gene expression upon a particular stimulus.

4.2. Classification of the etoposide-responsive genes

Among the 12 genes analyzed by Northern, eight genes are induced, three are repressed and one has no change by etoposide. Among the inducible genes, two (Waf-1 and PCNA) are previously known as p53 downstream targets and two (GPX and S100A2) appear to be novel p53 regulatory genes [21,22]. Induction of ODC, TGFβ-RII and MT-2 appears to be p53-independent or secondary effects of the p53-induction. Among the etoposide-repressed genes, HnRNP1 and tubulin β1 could be subjected to p53 regulation, since they are also repressed in a p53-induced apoptosis model detected by SAGE technology [24]. Since p53 inhibits the transcription of several cellular and viral promoters that lack a p53 binding site [25], more extensive work is needed to identify those subjected to p53 down-regulation.

4.3. Potential signalling pathways mediating etoposide-induced apoptosis

Based upon results obtained from this study, we propose here the potential signalling pathways that could mediate etoposide-induced apoptosis. As shown in Fig. 3, DNA damage induced by etoposide activates p53 which, in turn, transactivates Waf-1/p21, PCNA, GPX and S100A2 to initiate p53-dependent signalling pathways. In addition, induction of ODC and TGFβ-RII, two genes that have been shown to mediate apoptosis [26,27], would initiate polyamine and TGFβ signalling pathways, respectively. All these signalling pathways converge and ultimately lead to apoptosis, although many details in each pathway need to be worked out. It is clear that the chip technology can contribute significantly to our understanding of the complex cell responses to various stimuli. Once identified, such information allows for testing of more rigorous hypotheses and suggests pursuit of increasingly focused experimental strategies. The ultimate identification of patterns of responsive genes for any given treatment of interest will have a profound influence in the target identification and validation in future drug discovery.

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