Identification of genes responsive to apoptosis in HL-60 cells[1]

Wei JIN[2], Le-feng QU[3], Ping MIN[2], Shan CHEN[2], Hong LI[4], He LU[2,4], Yong-tai HOU[2, 5]

[2]*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203;*
[3]*Department of Vascular Surgery & Institute of Vascular Surgery of PLA, Changhai Hospital, Shanghai 200433;*
[4]*Le pôle franco-chinois du science du vivant et génomique, Ruijin Hospital, Shanghai 200025, China*

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ABSTRACT

AIM: To identify genes responsive to apoptosis in HL-60 cells treated by homoharringtonine. **METHODS:** cDNA microarray technology was used to detect gene expression and the result of microarrays for genes (TIEG and VDUP1) was confirmed by Northern analysis. **RESULTS:** Seventy-five individual mRNAs whose mass changed significantly were identified. Among these genes (25 were up-regulated and 50 were down-regulated), most are known related to oncogenes and tumor suppressor. Some genes were involved in apoptosis signaling pathways. **CONCLUSION:** TGFβ and TNF apoptosis signaling pathways were initiated during apoptosis in HL-60 cells. TIEG and VDUP1 play important roles in mediating apoptosis.

INTRODUCTION

Homoharringtonine (HHT) is a cytotoxic alkaloid isolated from the evergreen tree *Cephalotaxus harringtonia* native to the southern provinces of China. It can stop cell cycle by blocking cell G1 into S phase and from G2 into M phase. Clinical studies have indicated that HHT is effective in treating acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS), but not acute lymphoblastic leukemia (ALL) nor solid tumors[1]. HHT has been used alone and in combination with interferon-alpha or low-dose cytarabine in late and early chronic phases of CML patients, with positive results[2].

HHT induced apoptosis in HL-60 cells when the cells were exposed to 1×10^{-7} mol/L HHT for 4 h. DNA extracted from treated cells showed a typical internucleosomal DNA degradation. This effect of HHT was shown to appear in a concentration- and time-dependent manner. These results suggest that antitumor mechanism of HHT is related to its apoptosis inducing activity[3]. In HL-60 cells, HHT predominantly inhibited protein synthesis compared to RNA and DNA synthesis[4].

In an attempt to identify genes responsive to HHT-induced apoptosis and to reveal apoptosis signaling pathways, cDNA microarray technology was used to simultaneously display changes of gene expression. In all, ten microarrays were screened and each array representing 14218 human genes. Among the 75 genes characterized, most are previously known as cancer-related genes. A few are novel genes that may be involved in apoptosis signaling pathways.

MATERIALS AND METHODS

Cell cultures Human leukemia HL-60 cells were

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[5] Correspondence to Dr Yong-tai HOU.
Phn 86-21-5080-6600, ext 2416. Fax 86-21-5080-6029.
E-mail ythou@mail.shcnc.ac.cn
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cultured in RPMI-1640 medium containing 10 % bovine serum in 95 % air and 5 % CO_2 at 37 ºC. For the experiment, HL-60 cells were incubated with 4.64 mg/L HHT (Beijing Union Pharmaceutical Factory) or RPMI 1640 medium alone for 15 min, 1 h, 3 h, 6 h, 24 h.

Detection of apoptosis DNA fragmentation The total cellular DNA was extracted from HL-60 cells untreated and treated with HHT for 15 min,1 h, 3 h, 6 h, 24 h by the method described by Slin and Stafford with some slight modifications[5]. In brief, cells were washed in phosphate-buffered saline (PBS) and lysed overnight at 37 ºC in lysis buffer containing Tris-HCl 10 mmol/L (pH 8.0), edetic acid 10 mmol/L, 0.4 % sodium dodecylsulfate, and proteinase K 100 mg/L. After complete digestion, saturated phenol was added to the cell lysates and mixed fully. Samples were then centrifuged for 5 min. Chloroform was added to the supernatant isolated from the previous step, mixed fully and centrifuged as above. Supernatant was mixed with 2.5-fold volume of absolute ethanol and NaCl at 0.2 mol/L final concentration for DNA precipitation. The DNA pellets were obtained by centrifugation for 10 min and then air-dried, dissolved in TE buffer containing RNase 0.5 g/L (Sigma Chemical Co) at 37 ºC for 30 min. Electrophoresis was performed on 1.5 % agarose gel. The DNA was visualized by UV illumination.

RNA isolation Total RNA from HL-60 cells was extracted according to the original Chomczynski method with slight modifications[6]. Cells were collected and homogenized in Solution D containing 1 % β-mercaptoethanol. After centrifugation, supernatant was extracted with phenol:chloroform (1:1) twice and acidic phenol: chloroform (5:1) once. The RNA from aqueous phase was precipitated by cold isopropanol and dissolved in deionized H_2O (Milli-Q). Messenger RNAs were purified using an Oligotex-dT mRNA Midi Kit (Qiagen, Inc, Carlsbad, CA).

Construction of microarray and probe preparation The microarray was constructed according to Brown's method (in: http://cmgm.stanford.edu/pbrown/protocols/index.html). The 14218 microarray consists of 14218 full-length or partial complementary DNAs (cDNAs) representing novel, known and control genes provided by United Gene Holdings, Ltd (1111 Zhongshan Bei Er Road, Shanghai, China). The known genes were selected from National Center for Biotechnology Information (NCBI) Unigene set and cloned into PBS plasmid vector. The control spots of non-human origin included rice U2 RNA gene (8 spots), Hepatitis C Virus (HCV) coat protein gene (8 spots) and spotting solution alone (32 spots). The cDNA inserts were amplified by PCR using universal primers to the plasmid vector sequences. All PCR products were examined by agarose gel electrophoresis to ensure the quality and the identity of the amplified clones as expected. The PCR products were dissolved in a buffer containing 3×0.15 mol/L NaCl and 0.015 mol/L sodium citrate (SSC) solution. The solution were spotted onto silylated slides (CEL Associates, Houston TX) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, Irvine, CA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, CA). Glass slides with spotted cDNA were then hydrated for 2 h in 70 % humidity, dried for 0.5 h at room temperature, UV cross-linked (65 mj /cm). They were further processed at room temperature by soaking in 0.2 % sodium dodecyl sulfate (SDS) for 10 min, distilled H_2O for 10 min, and 0.2 % sodium borohydride ($NaBH_4$) for 10 min. The slides were dried and ready for hybridization.

The fluorescent cDNA probes were prepared through reverse transcription of the isolated mRNAs and then purified according to Schena *et al*[7,8]. The mRNA samples from the control cells were labeled with Cy3-dUTP (Amersham Pharmacia Biotech) and those from treated cells with Cy5-dUTP (Amersham Pharmacia Biotech). The two color probes were then mixed, precipitated with ethanol and dissolved in 20 μL of Hybridization Solution (5×SSC, 0.4 % SDS, 50 % formamide and 5×Denhardt's Solution).

Hybridization and washing of microarray Microarrays were pre-hybridized with Hybridization Solution containing 0.5 g/L denatured salmon sperm DNA at 42 ºC for 6 h. Fluorescent probe mixtures denatured at 95 ºC for 5 min were applied onto the pre-hybridized microarrays under cover glasses. After the microarrays were hybridized at 42 ºC for 15-17 h, they were washed at 60 ºC for 10 min each in solutions of 2×SSC and 0.2 % SDS; 0.1×SSC and 0.2 % SDS; 0.1×SSC, and then dried at room temperature.

Detection and analysis of microarray The microarrays were scanned with a ScanArray 3000 (GSI Lumonics, Bellerica, MA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using ImaGene 3.0 software (BioDiscovery, Inc, Los Angeles, CA). The intensities of each spot at the two wavelengths represent the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Ratio of Cy5 to Cy3 was computed

for each location on each microarray. Overall intensities were normalized with a correction coefficient obtained using the ratio of 40 housekeeping genes (list of these genes is available at http://www.biodoor.com/). Genes were identified as differentially expressed if the ratio of Cy5/Cy3 was >2 or <0.5. To minimize artifacts arising from low expression values, only genes with raw intensity values for both Cy3 and Cy5 of > 800 counts were chosen for differential analysis.

Northern analysis RNA (Northern) blots were carried out with mRNA extracted from the control and treated cells. The probes were labeled with ^{32}P (Beijing Furi bio-technology Ltd) using the random primer method. The blots were scanned with a cyclone instrument (Molecular Dynamics, Sunnyvale, CA), and the data were analyzed with Parkard 3.0 software (Packard Instruments, Meriden, CT).

RESULTS

HHT induced apoptosis in HL-60 cells Apoptosis induced by HHT in HL-60 cells was examined (Fig 1). DNA agarose gel electrophoresis showed that HL-60 cells presented the typical DNA ladder pattern of apoptosis after treatment with HHT 4.64 mg/L for 6 h and 24 h.

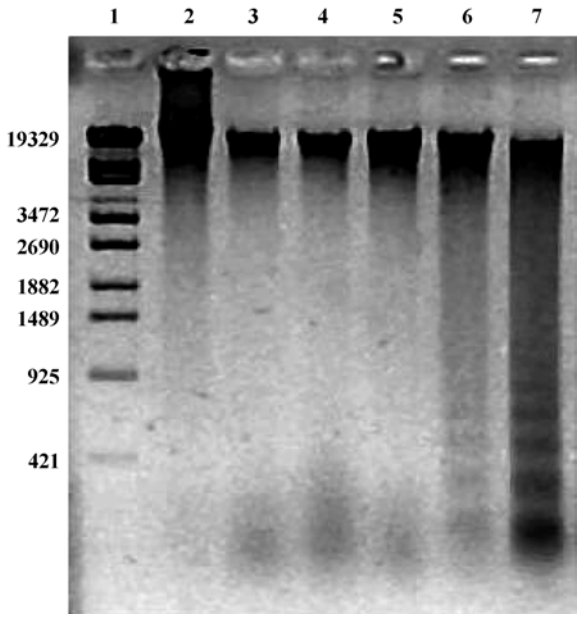

Fig 1. Patterns of DNA fragmentation during HHT induced apoptosis in HL-60 cells. 1) Lambda DNA/Eco130I (StyI) marker (bp); 2) untreated cells; 3) HHT 4.64 mg/L, 15 min; 4) HHT 4.64 mg/L, 1 h; 5) HHT 4.64 mg/L, 3 h; 6) HHT 4.64 mg/L, 6 h; 7) HHT 4.64 mg/L, 24 h.

cDNA microarray analysis In order to identify both early and late apoptosis-responsive genes, mRNA was isolated from cells treated with HHT for 15 min, 1 h, 3 h, 6 h, and 24 h and subjected to microarray hybridization. RNA samples from cells treated with RPMI 1640 medium were used as the control. A total of ten microarrays were screened. Two microarrays were screened for each parallel mRNA sample at each time point. The hybridization results from all ten microarrays were compiled and sorted on the basis of fold change compared to control cells. The average ratio of the two microarrays screened at each time point was adopted. Genes that displayed approximately two-fold or greater changes were scored as significant changes. Seventy-five mRNA species out of the 14218 genes were identified by these criteria. Of these 75 mRNA species, 25 were up-regulated and 50 were down-regulated at different time points (Tab 1).

Confirmation of HHT-induced genes by Northern analysis To confirm the results obtained from the microarray hybridization, we used the same batch of mRNA and performed Northern analysis with two HHT-induced genes: TIEG and VDUP1. As shown in Fig 2 and Fig 3, expression patterns for two genes correlate with the microarray results with slight difference. This may reflect more stringent hybridization conditions used for chip hybridization. The threshold we set for inclusion of HHT induced genes in the DNA chip analysis is, therefore, valid in two genes examined.

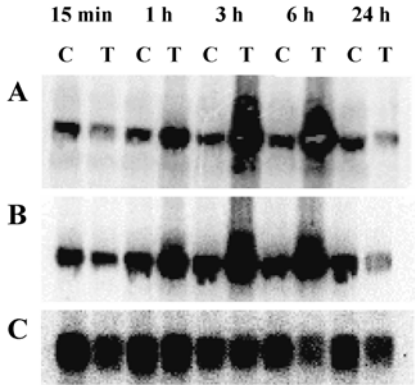

Fig 2. Northern analysis of (A) TIEG and (B) VDUP1. (C) β-ACTIN was used as an internal reference. Each lane contained 2 μg of mRNA isolated from HL-60 cells (C, control; T, treated with HHT) at different time.

Tab 1. List of differentially expressed genes responsive to HHT-induced apoptosis.

Symbol	GenBank	15 min ratio	1 h ratio	3 h ratio	6 h ratio	24 h ratio	Gene name
TIEG*	NM_005655	1.54	4.41	12.45	29.21	1.00	Transcription factor; TGFB inducible early growth response
ACVRL1*	NM_000020	2.91	7.83	8.549	0.55	0.47	Activin A receptor type II-like 1
PPP1CA*	NM_002708	1.53	2.28	4.27	5.64	1.48	Protein phosphatase 1, catalytic subunit, alpha isoform
IMMT*	NM_006839	0.95	0.48	2.21	5.82	0.88	Inner membrane protein, mitochondrial (mitofilin)
BRD2*	NM_005104	1.39	2.63	4.68	6.48	1.68	Bromodomain containing 2
NFKBIA*	NM_020529	1.70	1.93	5.73	8.77	1.45	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
DUSP6*	NM_001946	0.73	2.26	2.23	3.92	0.47	Dual specificity phosphatase 6
NFIL3*	NM_005384	0.64	1.37	2.01	3.44	0.56	Nuclear factor, interleukin 3 regulated
RAB14*	NM_016322	0.60	0.38	1.26	3.24	0.89	RAB14, member RAS oncogene family
VAV1*	NM_005428	0.98	0.47	1.51	5.07	1.15	Vav 1 oncogene
SLC3A2*	AB018010	1.48	1.57	4.82	7.65	1.00	Solute carrier family 3, member 2
TNFAIP3*	NM_006290	1.56	1.78	2.34	5.61	1.33	Tumor necrosis factor, alpha-induced protein 3
RBM4*	NM_002896	1.12	1.16	2.91	5.54	1.03	RNA binding motif protein 4
TAGLN2	D21261	1.15	0.44	1.29	3.85	0.99	Transgelin 2
FAP48	U73704	1.36	2.31	2.64	3.67	0.94	FKBP-associated protein
PPIE	AF042385	0.88	0.49	1.39	3.60	0.73	Peptidylprolyl isomerase E (cyclophilin E)
RRM1*	X59543	0.97	0.38	1.02	3.42	0.71	Ribonucleotide reductase M1 polypeptide
ABH	NM_006020	1.09	2.07	2.68	3.25	1.08	Alkylation repair; alkB homolog
HLA-A	D32129	0.61	0.29	0.79	2.89	0.35	Major histocompatibility complex, class I, A
M9	NM_004690	1.46	2.35	2.26	2.81	1.57	Muscle specific gene
ATP5G2	X69908	0.62	0.27	0.96	2.58	0.44	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 2
DDX21*	U41387	0.94	0.32	1.36	2.10	0.79	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21
TKT	L12711	0.50	0.34	0.81	2.02	0.33	Transketolase (Wernicke-Korsakoff syndrome)
VDUP1*	S73591	0.65	1.86	6.03	11.81	0.35	Thioredoxin interacting protein
C-MYC*	K02276	0.82	2.85	0.62	0.30	0.42	C-MYC, oncogene
IDH3B	AK001905	0.87	0.38	0.76	1.89	0.43	Isocitrate dehydrogenase 3 (NAD^+) beta
LAMR1	U43901	0.57	0.41	1.02	1.80	0.31	Laminin receptor 1 (ribosomal protein SA)
RPS9	AL080243	0.52	0.33	0.79	1.78	0.35	Ribosomal protein S9
FDPS	D14697	0.58	0.43	0.83	1.74	0.46	Dimethylallyltranstransferase (geranyltranstransferase)
DLEC1	AL137706	0.40	0.22	0.65	1.44	0.44	Deleted in lung and esophageal cancer 1
GNB2L1	M24194	0.49	0.69	0.73	1.41	0.28	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1
SAT	AL050290	0.33	0.69	0.93	1.40	0.26	Spermidine/spermine N1-acetyltransferase
SLC25A6	J03592	0.45	0.44	0.69	1.15	0.29	Solute carrier family 25, member 6
MVD	NM_002461	0.47	0.50	0.72	1.15	0.30	Mevalonate (diphospho) decarboxylase
RPL8*	Z28407	0.46	0.47	0.58	0.98	0.42	Ribosomal protein L8
BC-2	AF042384	0.38	0.49	0.52	0.96	0.33	Putative breast adenocarcinoma marker
HIP2	U58522	0.43	0.43	0.56	0.91	0.38	Huntingtin interacting protein 2
PSMB5	D29011	0.44	0.47	0.49	0.85	0.34	Proteasome (prosome, macropain) subunit, beta type, 5
YWHAE	U54778	0.41	0.58	0.49	0.82	0.33	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
MDH1	D55654	0.42	0.46	0.53	0.80	0.27	Malate dehydrogenase 1, NAD (soluble)
TAF7	U18062	0.49	1.07	0.71	0.78	0.40	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor
EIF3S5	U94855	0.36	0.82	0.67	0.80	0.29	Eukaryotic translation initiation factor 3, subunit 5 epsilon
SNRPA1	NM_003090	0.40	0.93	0.67	0.77	0.31	Small nuclear ribonucleoprotein polypeptide A'
RPS3A	M84711	0.46	0.74	0.51	0.76	0.32	Ribosomal protein S3A
SLC25A5	J02683	0.46	1.08	0.74	0.74	0.29	Solute carrier family 25, member 5
ACLY	X64330	0.49	0.89	0.61	0.73	0.32	ATP citrate lyase
SGCB	U31116	0.37	0.74	0.54	0.65	0.27	Sarcoglycan, beta (43kD dystrophin-associated glycoprotein)
ANP32B	NM_006401	0.46	0.73	0.45	0.64	0.27	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
FEN1	AC004770	0.65	0.47	0.45	0.64	0.34	Flap structure-specific endonuclease 1
NCL	M60858	0.53	0.74	0.40	0.63	0.24	Nucleolin

Symbol	GenBank	15 min ratio	1 h ratio	3 h ratio	6 h ratio	24 h ratio	Gene name
TRA1	X15187	0.55	0.49	0.41	0.60	0.27	Tumor rejection antigen (gp96) 1
SSB	X69804	0.56	1.07	0.55	0.59	0.41	Sjogren syndrome antigen B (autoantigen La)
PSMA2	D00760	0.72	1.20	0.46	0.59	0.29	Proteasome (prosome, macropain) subunit, alpha type, 2
DAD1*	NM_001344	0.39	1.01	0.47	0.59	0.23	Defender against cell death 1
RPL32*	X03342	0.42	1.14	0.40	0.58	0.20	Ribosomal protein L32
RPL10A*	AL022721	0.40	0.92	0.48	0.56	0.24	Ribosomal protein L10a
RAC1*	D25274	0.53	0.98	0.44	0.56	0.31	Ras-related C3 botulinum toxin substrate 1
PCNA*	M15796	0.64	0.75	0.44	0.26	0.51	Proliferating cell nuclear antigen
ARPC2	U50523	0.52	1.26	0.42	0.55	0.24	Actin related protein 2/3 complex, subunit 2 (34 kD)
DAXX*	NM_001350	0.58	0.81	0.47	0.55	0.60	Death-associated protein 6
NME2	M36981	0.40	0.80	0.42	0.54	0.47	Non-metastatic cells 2, protein (NM23B) expressed in
GRIN2B	NM_000834	0.62	0.65	0.77	0.53	0.34	Glutamate receptor, ionotropic, *N*-methyl *D*-aspartate 2B
CALM2	D45887	0.47	1.14	0.44	0.50	0.26	Calmodulin 2 (phosphorylase kinase, delta)
LBR	L25931	1.04	0.97	0.35	0.49	0.42	Lamin B receptor
UAP1	AB011004	0.51	0.96	0.54	0.47	0.41	UDP-*N*-acteylglucosamine pyrophosphorylase 1
LDOC1	AB019527	0.44	1.16	0.34	0.44	0.31	Leucine zipper, down-regulated in cancer 1
APT6M8-9	AL049929	0.38	0.63	0.38	0.40	0.39	ATPase, H^+ transporting, lysosomal interacting protein 2
HSPE1	U07550	0.95	1.14	0.33	0.39	0.44	Heat shock 10kD protein 1 (chaperonin 10)
PP	AB026723	0.44	1.49	0.56	0.38	0.26	Pyrophosphatase (inorganic)
INSIG1	U96876	0.49	0.48	0.45	0.37	0.40	Insulin induced gene 1
MTHFD2	X16396	0.38	0.58	0.32	0.32	0.25	Methenyltetrahydrofolate cyclohydrolase
H3F3A	M11353	1.03	1.00	0.28	0.29	0.61	H3 histone, family 3A
LNPEP	AJ131023	0.97	1.10	0.46	0.25	0.70	Leucyl/cystinyl aminopeptidase
IGFBP7	X63563	0.81	0.63	0.24	0.23	0.57	Insulin-like growth factor binding protein 7
MGC2668	AK001880	0.61	0.82	0.25	0.23	0.43	Hypothetical protein MGC2668

Note: the gene labeled means the ones need explanation for the table.

DISCUSSION

Previous researches have shown that apoptosis of HL-60 cells could be induced when treated by 1 mg/L[9] or 10 mg/L HHT[10]. Our experiments showed that apoptosis of HL-60 cells could be induced after treatment with 4.64 mg/L HHT for 6 h and 24 h.

In an attempt to identify genes whose expression changed during HHT-induced apoptosis in HL-60 cells, we utilized cDNA microarray technology to obtain an overall profile of gene expression.

Among the up-regulated genes, TIEG and VDUP1 overexpressed more significantly than other induced genes. It was reported that overexpression of TIEG or VDUP1 could induce apoptosis in some kinds of cells. TIEG is a early growth response product induced by transforming growth factor-beta. Overexpression of TIEG mimics TGFβ action and play a role in TGFβ-induced inhibition of cell proliferation and apoptosis in human osteoblast cells and pancreatic carcinoma cells

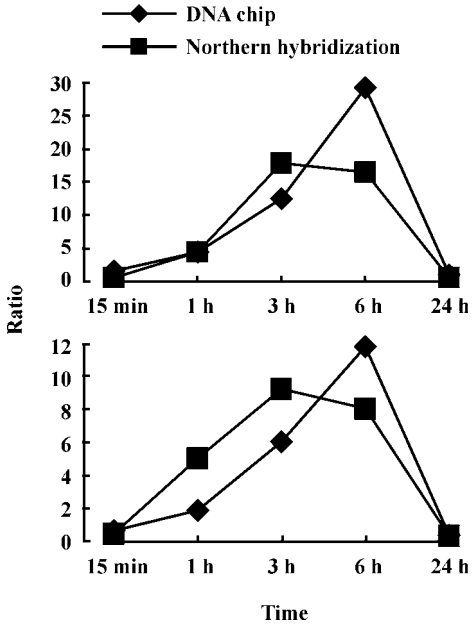

Fig 3. Expression patterns of (A) TIEG and (B) VDUP1 and correlation between Northern analysis and DNA chip results.

and more recently in epithelial and liver cancer cells[11-14]. VDUP1 is vitamin D3 up-regulated gene 1, VDUP1 mediated oxidative stress via suppressing the thioredoxin (TRX) function. TRX has functions in defense against oxidative stress and control of growth and apoptosis. VDUP1 acts as an endogenous inhibitor of TRX by interacting with the catalytic active center of TRX to induce apoptosis and sensitize cells to oxidative stress-mediated apoptosis[15,16].

Among the other up-regulated genes, C-MYC was induced earlier. It was reported that the induction of C-MYC may lead to apoptosis[17]. ACVRL1 is type I cell-surface receptor for the transforming growth factor-beta superfamily of ligands[18]; Induction of TIEG and ACVRL1 indicated TGFβ signaling pathways was initiated. TNFAIP3 is tumor necrosis factor alpha-induced protein 3[19], but it encodes a protein that inhibits apoptosis[20]; NFKBIA is a nuclear factor for kappa light chain gene enhancer in B-cells. It inhibits NF-κB activity by binding the rel domain of NF-κB components[21,22]. Induction of TNFAIP3 and NFKBIA indicated TNF signaling pathways was initiated. As we know, NFκB may resist apoptosis, the induction of NFKBIA in our experiment would inhibit NFκB activity by binding the rel domains of NF-κB components to induce apoptosis. PPP1CA is catalytic subunit of protein phosphatase 1 which regulates mitosis and is a putative tumor suppressor[23]; VAV1 is a member of the Dbl family of guanine nucleotide exchange factors (GEF) for the Rho family of GTP binding proteins[24]. This particular GEF has been identified as the specific binding partner of Nef proteins from HIV-1. Coexpression and binding of these partners initiates profound morphological changes, cytoskeletal rearrangements and the JNK/SAPK signaling cascade. DDX21 with a conserved motif Asp-Glu-Ala-Asp (DEAD), is putative RNA helicase. This gene encodes a DEAD box protein, which is an antigen recognized by autoimmune antibodies from a patient with watermelon stomach disease[25]. This protein unwinds double-stranded RNA, folds single-stranded RNA, and may play important roles in ribosomal RNA biogenesis, RNA editing, RNA transport, and general transcription[26]. RRM1 is one of several genes located in the imprinted gene domain of 11p15.5, an important tumor-suppressor gene region. Alterations in this region have been associated with the Beckwith-Wiedemann syndrome, Wilms tumor, rhabdomyosarcoma, adrenocrotical carcinoma, lung, ovarian and breast cancer. This gene may play a role in malignancies and disease that involve this region. It maybe a tumor suppressor and is the most likely candidate gene with metastasis suppressor function[27,28]. NFIL3 is a nuclear factor, which activates IL-3 gene expression. It binds to regulatory sequences in the promoters of adenovirus E4, gamma interferon (IFNG), and interleukin 3 (IL-3) genes[29]. DUSP6 is dual specificity phosphatase 6[30]. It selectively dephosphorylates and inactivates MAP kinase, which is associated with cellular proliferation, differentiation and apoptosis[31]; BRD2 is bromodomain-containing 2. It encodes a mitogen-activated kinase, which localizes to the nucleus, maybe part of a signal transduction pathway involved in growth control[32]. It may play an important role in human development[33]; IMMT encodes inner membrane protein[34]. It has a sequence encoding a new human motor protein[35]; RBM4 is RNA binding motif protein 4, a putative RNA recognition motif (RRM)-type RNA-binding protein, with a retroviral-type zinc finger, similar to D melanogaster lark RNA-binding protein[36]. SLC3A2 is one of the earliest expressed antigens on the surface of activated human lymphocytes[37].

Among the down-regulated genes, PCNA was originally defined as a nuclear protein whose appearance correlated with the proliferation state of the cell. It is now known to be a co-factor of DNA polymerase delta and to be necessary for DNA synthesis and cell cycle progression[38]. It was also known as p53 downstream target[39]. RAC1 is a member of the Rho family of small GTPases involved in signal transduction pathways that control proliferation, adhesion, and migration of cells during embryonic development and invasiveness of tumor cells[40]. DAD1, the defender against apoptotic cell death, was initially identified as a negative regulator of programmed cell death in the temperature sensitive tsBN7 cell line. The DAD1 protein disappeared in temperature-sensitive cells following a shift to the nonpermissive temperature, suggesting that loss of the DAD1 protein triggered apoptosis[41,42]. The down-regulation of DAD1 indicated apoptosis was induced. RPL10A, RPL32 and RPL8 encode ribosomal proteins, down-regulation of these genes indicated the function of ribosomal proteins was disturbed.

DAXX is a death-associated protein 6. It binds to the Fas (fatty acid synthase) cell surface receptor and activates apoptosis through the Jun N-terminal kinase (JNK) signal transduction pathway[43]. DAXX mRNA level did not change significantly in this experiment suggesting Fas signaling pathway might not be initiated.

In conclusion, the cDNA microarray analysis indicates a variety of gene expression changes responsive to HHT-induced apoptosis. Most changed genes are related to cancer and cell apoptosis and proliferation. Our results demonstrate TGFβ and TNF apoptosis signaling pathways were initiated and Fas signaling pathway was not initiated. Some genes could resist or defense against apoptosis, when these genes were down-regulated, apoptosis could be induced easily. Overexpression of TIEG and VDUP1 could induce apoptosis in some kinds of cells. TIEG and VDUP1 were up-regulated most significantly in this experiment which were important factors resulted in apoptosis. Other genes, such as BRD2, ACVRL1 and RBM4 were up-regulated more or less. They may be involved in apoptosis signaling pathways. Future studies with more cell lines and primary tumors are required to confirm and supplement these data. Characterization of these HHT-induced genes may lead to the identification of novel targets for the discovery of anticancer drugs.

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