Proteome Profiling of Arsenic Trioxide-treated Human Hepatic Cancer Cells

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Abstract. *Background: Arsenic trioxide (As_2O_3), a major compound in traditional Chinese medicine, is known to be an effective anticancer agent in acute promyelocytic leukemia (APL). The effects of As_2O_3 on human hepatocellular carcinoma (HCC) SK-Hep-1 cells were studied employing proteomics-based methodologies. Materials and Methods: Using 1-dimensional electrophoresis (1DE) and liquid chromatography electrospray ionization quadruple time-of-flight analysis, the whole proteomes of the control and As_2O_3-treated cells were profiled. Results: In all, 207 and 62 proteins, which were specifically found in control and As_2O_3-treated cells, respectively, were classified with their biological processes by gene ontology (GO) annotation. The GO data indicated that 16 proteins were closely associated with apoptotic mechanisms. As_2O_3-induced DNA damage and oxidative stress that accompanied apoptosis in SK-Hep-1 cells were observed using comet assay and 5- and-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate fluorescence microscopy, respectively. Conclusion: The anticancer activities of As_2O_3 may be mediated by DNA damage- and reactive oxygen species-induced apoptotic mechanisms which involve the proteins identified in this study.*

Hepatocellular carcinoma (HCC) is a fatal disease with a 5-year survival rate of 5% in HCC patients with poor prognosis (1). HCC treatment involves the use of drugs such as doxorubicin and methotrexate. The side-effects of these drugs, however, are a cause of concern since they have been linked to hepatitis and heart damage in HCC patients. Therefore, the discovery of new effective drugs is essential for the treatment of HCC (2).

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Recently, the search for new compounds for cancer therapy has been the focus of numerous studies, many of which have focused on compounds that have been widely used in traditional medicine (3). Arsenic trioxide (As_2O_3) is a major compound in traditional Chinese medicine used to cure hemorrhoids, acute ulcerative gingivitis, and asthma (2). Nevertheless, it has not been considered as a therapeutic agent because of its toxic side-effects when used long-term (4). However, arsenic compounds have been reevaluated for their anticancer properties (2, 5). It has been reported that As_2O_3 predominantly induced apoptosis in acute promyelocytic leukemia (APL) cells (6, 7), which was mediated by increased levels of intracellular reactive oxygen species (ROS), depolarization of mitochondrial membranes and DNA damage (6-8). The apoptotic effects of As_2O_3 in APL have led investigators to propose that As_2O_3 may also induce apoptotic mechanisms in other types of cancer cells (9-11). However, little is known about the molecular mechanisms of As_2O_3-induced apoptosis in these cancer cells.

Previous studies have shown that As_2O_3 significantly inhibited cell proliferation and induced apoptosis in HCC cell lines (12-14). Oketani *et al*. showed that As_2O_3 inhibited cell proliferation and induced apoptosis in Huh-7, HepG2 and SK-Hep-1 cells as demonstrated by the increased appearance of sub-G_1 cells after treatment with As_2O_3 (13). The sensitivity of HCC cells to As_2O_3 was also shown to be inversely related to intracellular glutathione (GSH) concentrations and GSH synthesis levels (13).

In this present study, the molecular mechanisms of As_2O_3-induced apoptosis were investigated in SK-Hep-1 cells using proteomics-based methodologies.

Materials and Methods

Cell culture and treatment. The human HCC cell line SK-Hep-1 was purchased from the Korean Cell Line Bank. SK-Hep-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and penicillin (100 μg/ml) and kept in a humidified atmosphere at 37˚C with 5% carbon dioxide. As_2O_3 was purchased from Sigma (Deisenhofen, Germany), prepared as a 10 mM stock solution dissolved in DMSO and stored at 4˚C. The SK-Hep-1 cells were seeded in 100-mm culture dishes at a density of 5 mM 6 cells.

As the cells reached 70% confluence, the medium was replaced by fresh medium containing As_2O_3 while the control cells were cultured in medium containing the same volume of DMSO without As_2O_3.

Cell proliferation assay. To assess the effect of As_2O_3 on cell viability, SK-Hep-1 cells (2×10^3) were seeded in a 48-well plate. After 24 h incubation, the cells were treated with different concentrations of As_2O_3 (0, 0.25, 0.75, 1, 2, 3, 5, 10 μM). After 24 h (or 48 h) of treatment, the medium was removed and each well was treated with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-5-diphenyl-tetrazolium bromide (MTT; Sigma) solution at 37˚C for 2 h, and solubilized using 100 μl of DMSO. The plate was then vortexed for 1 h and the level of formazan was measured by a spectrophotometer at 575 nm.

1-dimensional electrophoresis (1DE). Protein expression profiles of SK-Hep-1 cells before and after treatment with 2 μM As_2O_3 for 48 h were observed by 1-DE analysis. The cells were harvested and lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The protein concentration of the lysates was determined by Bradford assay. For 1-DE, 50 μg of proteins were collected from the lysates of the control and As_2O_3-treated cells, and loaded into the 10% SDS polyacrylamide gels. The proteins were separated at 50 V for 30 min using a Mini-Protean II system (Bio-rad, Hercules, CA, USA). After electrophoresis, the polyacrylamide gel was stained using a Coomassie Brilliant Blue G-250 reagent (Bio-Rad). Each of the stained gels was sliced into 15 pieces and used for further mass spectrometry/mass spectrometry (MS/MS) analysis.

Liquid chromatography electrospray ionization quadruple time-of-flight (LC-ESI-Q-TOF). The LC MS/MS system comprised a Surveyor MS pump (Thermo Electron, San Jose, CA, USA), a Spark auto sampler (Spark, Emmen, the Netherlands), and a Finnigan LTQ linear ion trap MS (Thermo Electron) equipped with NSI sources. An aliquot of 10 μl of tryptic digested peptides was injected for the LC-MS/MS analysis. Digested samples were also injected directly into a peptide CapTrap cartridge for concentration and desalting and applied to a reversed phase (RP) column that was packed in-house with 5 μm, 300 Å pore size C18. The peptides were then separated on an RP column. The mobile phase A (H_2O) and B (acetonitrile) solutions contained 0.1% v/v formic acid. The flow rate was maintained at 200 nl/min. The gradient was started at 5% B and a linear gradient up to 60% B was achieved in 49 min, then ramped to 80% B in 5 min and to 100% A in the next 15 min.

The MS was operated in a data-dependent mode (m/z 300-1800) in which each full MS scan was followed by five MS/MS scans where the five most abundant peptide molecular ions were dynamically selected from the prior scan for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of the heated capillary and electrospray voltage were 195˚C and 1.9 kV, respectively.

Gene ontology (GO) annotation. The LC-ESI-Q-TOF data of total proteins have Uniprot accession numbers (Swiss-Prot numbers or Translated EMBL numbers). GO annotation was then assigned to the Uniprot accession numbers using the QuickGO web tool (http://www.ebi.ac.uk/ego/). Each Uniprot accession number was assigned to three categories: biological process, molecular function, and cellular component (15, 16). If a single protein was annotated by several processes, such as functions or components, a representative of such annotations was reflected in the data.

Comet assay. Comet assay (Trevigen, Gaithersburg, MD, USA) was performed to observe DNA damage of the As_2O_3-treated cells compared to that of the control. The cells treated with 2 μM As_2O_3 (or 1% DMSO) for 48 h were harvested using a scraper and washed once with ice-cold phosphate-buffered saline, then re-suspended to a density of 3×10^5 cells/ml. Aliquots (5 μl) of this suspension were added to 500 μl low melting agarose; subsequently, 75 μl of this were poured onto the provided microscope slides. The agarose solution was allowed to set at 4˚C for 30 min and the slides were then immersed in lysis solution at 4˚C for 1 h to dissolve cellular proteins and lipids. After placing in alkali solution for 1 h at room temperature in a dark environment, the slides were immersed in 1× Tris-borate-EDTA (TBE) buffer for 5 min twice and transferred to a horizontal electrophoresis apparatus containing 1× TBE buffer for 10 min at 25 V in the dark. The slides were gently washed twice in distilled water. After staining with Hoechst 33342 solution (Invitrogen), the slides were examined by fluorescence microscopy (Axiovert 200; Zeiss Co, Oberkochen, German) and analyzed using Comet Assay IV (Perceptive Instuments Ltd. Suffolk, UK). Three comet parameters were evaluated for each image: tail lenth (μm), tail intensity (DNA %), and tail moment.

ROS detection. To investigate the effect of As_2O_3 on intracellular ROS production in the SK-Hep-1 cells, fluorometric assay using 5- and-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was used as a probe for ROS detection. SK-Hep-1 cells (1×10^6) were plated in 60-mm culture dishes, and treated with 0.1% DMSO or 2 μM As_2O_3 for 48 h. After treatment, the cells were rinsed with HBSS solution and incubated with a medium containing 20 μM DCF-DA for 15 min at 37˚C in the dark. The DCF-DA loaded cells were rinsed with HBSS solution and observed by fluorescence microscopy. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, and fluorescent images were taken at 5-s intervals.

Results

Antiproliferative activity of As_2O_3. The MTT assay showed that As_2O_3 inhibited the growth of the SK-Hep-1 cells in a dose- and time-dependent manner (Figure 1). While a high dose of As_2O_3 (more than 2 μM) caused noticeable cytotoxicity within 1 day, a low dose of As_2O_3 (less than 1 μM) did not significantly inhibit the cell growth. The half maximal inhibitory concentration (IC_{50}) value of As_2O_3 on SK-Hep-1 was about 4.5 μM for 24 h treatment and 2.3 μM for 48 h treatment.

Classification of identified proteins by GO analysis. Using LC-ESI-Q-TOF, in all, 733 and 558 proteins were identified from the control and As_2O_3-treated cells, respectively. Out of these, 471 proteins were found to be expressed in both groups, while 262 and 94 proteins were specifically expressed in the control or As_2O_3-treated cells, respectively. Based on GO annotation, 207 and 62 proteins were annotated and classified by biological processes, such as transport, translation, carbohydrate metabolic process, DNA replication, RNA splicing, transcription, cell proliferation, signal transduction (including Ras-related signal transduction), apoptosis and response to oxidative stress (Table I). The GO annotation data indicated

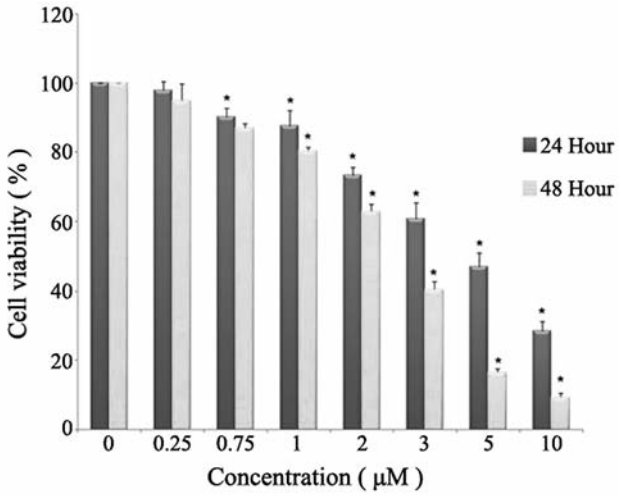

Figure 1. *Effect of As_2O_3 on viability of SK-Hep-1 cells determined using MTT assay. Mean±SD of three independent experiments. *p<0.01 compared to the control.*

that 94 (76 down-regulated proteins, 18 up-regulated proteins) of the total identified proteins were associated with the proliferation of cells, classified as translation, DNA replication, transcription, cell proliferation, apoptosis, Ras-related signal transduction, cell growth, oxidation reduction, response to oxidative stress, DNA repair and anti-apoptosis. Sixteen proteins were closely associated with DNA damage-induced apoptosis and regulation of intracellular ROS (Table II).

Effect of As_2O_3 on DNA damage and intracellular ROS level. Using the comet assay the As_2O_3-treated cells showed significant DNA strand breaks compared to the control (Figure 2, Table III). DCF-DA fluorescent microscopy showed that the production of intracellular ROS was significantly increased after treatment with As_2O_3, and indicated that the morphology of the cells was noticeably changed after the treatment with As_2O_3 (Figure 3).

Discussion

Many studies have indicated that the anticancer activities of As_2O_3 in HCC are significantly related to the apoptotic mechanisms, which are mediated by DNA damage (17, 18) and increased oxidative stress (19-21). In the present study, As_2O_3 inhibited cell proliferation in SK-Hep-1 (Figure 1). Among 269 differentially expressed proteins in the control and As_2O_3-treated cells, 94 were closely related to the anticancer activities of As_2O_3, including translation, DNA replication, transcription, cell proliferation, apoptosis, Ras-related signal transduction, cell growth, oxidation reduction, response to oxidative stress, DNA repair and anti-apoptosis. Interestingly, 16 proteins were closely related to the apoptotic mechanisms mediated by DNA damage and increased oxidative stress (Table II). The anticancer activity of As_2O_3 was associated with increased DNA damage (Figure 2) and ROS production (Figure 3) as observed by comet assay and ROS detection, respectively.

DNA damage-induced apoptosis is considered as an important effect of anticancer activity of As_2O_3 (17, 18). In this study, ten of the total identified proteins were directly associated with the induction of apoptosis: mitochondrial 28S

Table I. *GO biological terms of identified proteins classified by their biological process.*

Biological process	Control specific (Down-regulated)	As_2O_3-treated specific (Up-regulated)	Total
Translation	24	9	33
Transport	23	10	33
Carbohydrate metabolic process	11	4	15
RNA splicing	11	3	14
DNA replication	12	0	12
Signal transduction	7	4	11
Transcription	9	2	11
Cell proliferation	7	3	10
Developmental process	2	6	8
Ubiqutin-dependent protein catabolitic process	5	3	8
Apoptosis	6	2	8
Protein folding	6	2	8
Lipid metabolic process	7	1	8
Nucleotide metabolic process	3	4	7
Protein modification process	5	2	7
Actin filament-based process	3	3	6
Ras-related signal transduction	6	0	6
Proteolysis	6	0	6
Cellular metabolic process	4	1	5
Response to stress	4	0	4
Ion transport	4	0	4
Cell motility	4	0	4
Cell adhesion	4	0	4
Cell growth	4	0	4
Electron transport	3	0	3
Immune response	3	0	3
Generation of precursor metabolite and energy	3	0	3
Cell differentiation	2	1	3
Oxidation reduction	2	1	3
Response to oxidative stress	2	1	3
Muscle contraction	2	0	2
DNA repair	2	0	2
Amino acid metabolic process	2	0	2
Anti-apoptosis	2	0	2
Cell shape	2	0	2
Phosphorylation	1	0	1
Exocytosis	1	0	1
Endocytosis	1	0	1
Digestion	1	0	1
ATP catabolic process	1	0	1
	207	62	269

Table II. *Apoptosis- and ROS-related proteins.*

Accession no.	Protein name	Biological process	Function (Swiss-Prot)	Regulation
P48637	Glutathione synthetase	Response to oxidative stress		Down
Q6ZR44	CDNA FLJ46672 fis, clone TRACH3009008, highly similar to thioredoxin reductase	Response to oxidative stress		Down
O95747	Serine/threonine-protein kinase OSR1	Response to oxidative stress	Regulates downstream kinases in response to environmental stress.	Up
P35270	Sepiapterin reductase	Oxidation reduction	Catalyzes the final one or two reductions in tetra-hydrobiopterin biosynthesis to form 5,6,7,8-tetrahydrobiopterin.	Down
P31930	Ubiquinol-cytochrome *c* reductase complex core protein 1, mitochondrial [Precursor]	Oxidation reduction	Component of the ubiquinol-cytochrome *c* reductase complex. This protein may mediate formation of the complex between cytochromes *c* and *c1*.	Down
P47985	Cytochrome b-c1 complex subunit Rieske, mitochondrial	Oxidation reduction	Component of the ubiquinol-cytochrome *c* reductase complex.	Up
P51398	28S ribosomal protein S29, mitochondrial	Apoptosis	Involved in mediating interferon-gamma-induced cell death.	Down
Q9NP92	28S ribosomal protein S30, mitochondrial	Apoptosis		Down
O95831	Apoptosis-inducing factor 1, mitochondrial	Apoptosis	Probable oxidoreductase that acts as a caspase-independent mitochondrial effector of apoptotic cell death.	Down
O75489	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial [Precursor]	Apoptosis	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase.	Down
O14737	Programmed cell death protein 5	Apoptosis	May function in the process of apoptosis.	Down
P67775	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Apoptosis	Modulates the activity of phosphorylase B kinase casein kinase 2, mitogen-stimulated S6 kinase, and MAP-2 kinase.	Down
O43324	Eukaryotic translation elongation factor 1 epsilon-1	Apoptosis	Positive modulator of ATM response to DNA damage.	Up
Q04760	Lactoyl glutathione lyase	Apoptosis	Catalyzes the conversion of hemi-mercaptal, formed from methylglyoxal and glutathione, to *S*-lactoylglutathione.	Up
P62195	26S protease regulatory subunit 8	Anti-apoptosis	Involved in the ATP-dependent degradation of ubiquitinated proteins.	Down
P06493	Cell division control protein 2 homolog	Anti-apoptosis	Plays a key role in the control of the eukaryotic cell cycle.	Down

ribosomal protein S29 (MRPS29), mitochondrial 28S ribosomal protein S30 (BM-047), mitochondrial apoptosis-inducing factor 1 (AIFM1), mitochondrial NADH dehydrogenase (ubiquinone) iron-sulfur protein 3 (NDUFS3), programmed cell death protein 5 (PDCD5), serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PPP2CA), eukaryotic translation elongation factor 1 epsilon-1 (MCA3), lactoyl glutathione lyase (LGUL), 26S protease regulatory subunit 8 (PSMC5) and cell division control protein 2 homolog (CDC2). MRPS29, BM-047, AIFM1, NDUFS3, PDCD5, PPP2CA, MCA3 and LGUL are regarded as apoptosis-inducing proteins, while PSMC5 and CDC2 are responsible for the inhibition of apoptotic mechanisms. Among the apoptosis-inducing proteins, the expression of MCA3 and LGUL was up-regulated after treatment with As_2O_3, while that of the other proteins was down-regulated. Moreover, the expression of PSMC5 and CDC2 was down-regulated after the treatment. This expression change suggested that the apoptotic mechanisms of As_2O_3 seem to be more directly related to the expression of these four proteins (MCA3, LGUL, PSMC5, and CDC2) than the other proteins. In addition, many studies have

Table III. *The comet assay result of control and As_2O_3-treated cells.*

Group	Comet parameters		
	Tail length (μm)	Tail intensity (DNA %)	Tail moment
Control	22.46±6.21	0.23±0.02	0.18±0.01
As_2O_3-treated cells	255.92±101.92	94.48±6.63	136.23±49.23

Mean±SD of three independent experiments.

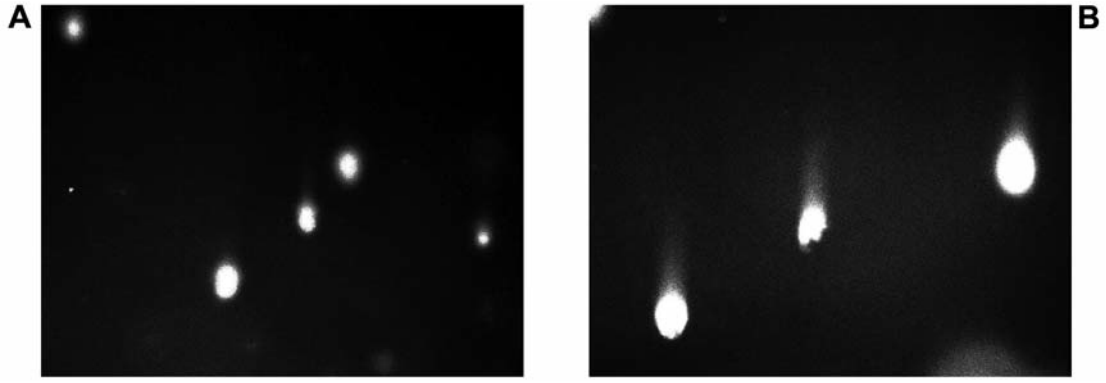

Figure 2. *As_2O_3-induced DNA damage in SK-HEP-1 cells, assessed by comet assay in control (A) and As_2O_3-treated cells (B). Representative images of three independent experiments.*

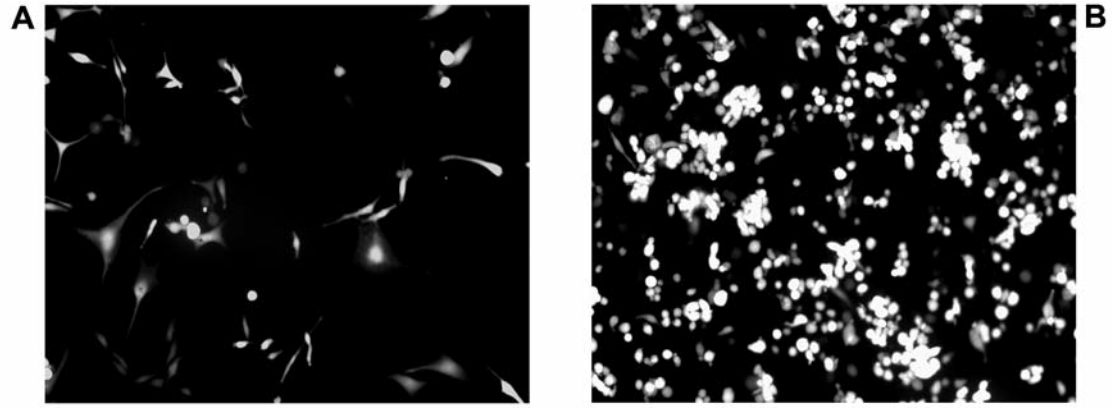

Figure 3. *As_2O_3-induced ROS in SK-Hep-1 cells using DCF-DA probe fluorescence microscopy. Control (A) and As_2O_3 treatment (B).*

shown that the apoptotic mechanisms of As_2O_3 are related to the change in CDC2 expression (22-26). Cai *et al*. showed that As_2O_3-induced apoptosis and mitotic arrest in NB4 cells, an acute promyelocytic leukemia cell line, accompanied the inhibition of CDC2 expression (26).

Several studies have suggested that the change in intracellular ROS level is related to the As_2O_3-induced apoptosis in HCC (19, 20). In this proteomics-based study, six proteins, glutathione synthetase (GSS), cDNA FLJ46672 fis clone TRACH3009008 (highly similar to thioredoxin reductase [Q6ZR44]), serine/threonine-protein kinase OSR1(OXSR1), sepiapterin reductase (SPR), ubiquinol-cytochrome *c* reductase complex core protein 1 (UQCRC1) and cytochrome *b-c*1 complex subunit Rieske (UQCRFS1), which are considered to be responsible for the control of the intracellular ROS level, were found. Interestingly, the molecular function of these six proteins is categorized as "oxidoreductase activity", which might be an important mechanism for the As_2O_3-induced oxidative stress. However, it appeared that only the up-regulated proteins (OXSR1, UQCRFS1) showed this oxidoreductase activity in the As_2O_3-treated cells. Down-regulation of the other proteins (GSS, Q6ZR44, SPR, and UQCRC1) might be related to the conditions for the production of ROS, which resulted in further apoptotic mechanisms. Moreover, several studies have shown that the depletion of GSS resulted in the decreased production of glutathione (GSH) (27, 28), which is one of the major antioxidant proteins that functions in the lungs against oxidative stress. A previous study (13) also showed that the sensitivity of SK-Hep-1 cells to As_2O_3 was increased after a decrease in the intracellular level of GSH. Thus, the decreased expression of GSS might be an important event in As_2O_3-induced apoptosis.

Overall, this proteomics-based study provides a better understanding of the As_2O_3-induced apoptotic mechanism, and suggests that As_2O_3 may have significant therapeutic value for the treatment of human solid tumors. Further studies need to be carried out focusing on the specific function of the identified proteins in As_2O_3-treated SK-Hep-1 cells.

In conclusion, many proteins identified in As_2O_3-treated SK-Hep-1 cells are associated with the anticancer activities of As_2O_3 and some are involved in apoptotic mechanisms. The effect of As_2O_3 is mediated by DNA damage and increased intracellular ROS level.

Acknowledgements

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