Toxicoproteomic evaluation of carbon nanomaterials *in vitro*

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22 Keywords

23 Toxicoproteomics; Chronic toxicity; Carbon nanotubes; Nanomaterials; *in vitro*

Carbon nanotubes (CNTs) have already been successfully implemented in various fields, and they are anticipated to have innovative applications in medical science. However, CNTs have asbestos-like properties, such as their nanoscale size and high aspect ratio (>100). Moreover, CNTs may persist in the body for a long time. These properties are thought to cause malignant mesothelioma and lung cancer. However, based on conventional toxicity assessment systems, the carcinogenicity of asbestos and CNTs is unclear. The reason for late countermeasures against asbestos is that reliable, long-term safety assessments have not yet been developed by toxicologists. Therefore, a new type of long-term safety assessment, different from the existing methods, is needed for carbon nanomaterials. Recently, we applied a proteomic approach to the safety assessment of carbon nanomaterials. In this review, we discuss the basic concept of our approach, the results, the problems, and the possibility of a long-term safety assessment for carbon nanomaterials using the toxicoproteomic approach.

Conventional Safety assessments of CNTs

During the past 10 years, many studies have examined the toxicity of CNTs *in vivo* and *in vitro* (Table 1). Cytotoxicity, cytokine production and oxidative stress occur when various types of cells are cultured with CNTs [1-5]. Pulmonary exposure to CNTs caused rats and mice to develop fibrosis, granulation and inflammation in their lungs [6-8]. Takagi et al. found that the intraperitoneal injection of CNTs into $p53^{-/-}$ mice caused malignant mesothelioma [9], and Poland et al. reported that CNTs exhibit asbestos-like pathogenicity in mice [10]. However, published replies to these reports questioned the appropriateness of the administration sites or CNT dosage [11, 12]. Moreover, Muller et al. found no carcinogenic response to CNTs placed in the peritoneal cavity of rats for 2 years [13]. Many factors may be responsible for these conflicting results, but we do not yet have sufficient information about the factors that contribute to CNT toxicity in rodents or in cell culture [6].

Biological responses to CNTs are affected by multiple properties that include length, shape (single-wall or multi-wall), fibrous surface area, aspect ratio and aggregability with or without the involvement of dispersion medium [10, 14-22]. Impurities, such as iron and polycyclic aromatic hydrocarbons, are introduced into CNTs by the production process. These impurities have intrinsic toxicities, and their interaction with CNTs in

56 cells can be cytotoxic [23-26]. Conventional methods suitable for the examination of
57 acute or subacute toxicity have been used to examine the properties of CNTs that
58 contribute to biological responses.

59 Mesothelioma caused by asbestos exposure cannot be reliably reproduced in a
60 conventional toxicity assay *in vitro*. Asbestos does not induce transformation of primary
61 human mesothelial cells in tissue culture. Rather, asbestos represents cytotoxicity that
62 leads the cell death to human mesothelial cells grown *in vitro* [27-29]. Therefore, it is
63 difficult to design an experiment that contains a positive control because the
64 mechanism for the development of mesothelioma remains unclear [30]. Essentially, the
65 present scientific evidence is insufficient to conclude the possible long-term toxic effects
66 of CNTs, such as development of mesothelioma.

67 Animal experiments used to evaluate chronic toxicity are also controversial. When
68 CNTs are greater than 5 μm in length, it is thought that they are too large to reach the
69 distal portions of the lungs in mice or rats [31]. In fact, there are few reports of
70 mesothelioma onset in rodents by asbestos exposure (especially particles ≥ 8 μm in
71 length ≤ 0.25 μm in width). Therefore, out of public concern, evaluating intraperitoneal
72 injection of CNTs for the development of lung mesothelioma, commonly seen in cases of
73 asbestos exposure, has been attempted. However, as mentioned, the effectiveness of this

74 method is not without controversy. Indeed, it is difficult to predict the long-term safety
75 of CNTs in humans based on the results of animal studies. Therefore, a *de novo*
76 procedure must be developed to evaluate the safety of long-term exposure to carbon
77 nanomaterials containing CNTs.
78
79 **Safety evaluation of carbon nanomaterials by proteomics**
80 Witzmann and Monteiro-Riviere have used a proteomic approach to study the
81 biological responses of human keratinocytes to multi-walled CNTs [32]. Their analysis
82 identified proteins related to metabolism, cell signaling and stress, as well as
83 cytoskeletal elements and vesicular trafficking components. Chang et al. used a
84 proteomic analysis to study the mechanism of ultrafine carbon black-induced lung
85 injury in mice [33]. They analyzed proteins in bronchoalveolar lavage fluid and
86 identified 33 proteins, including leukemia inhibitory factor receptor (LIFR) and
87 epidermal growth factor receptor (EGFR). In these experiments, the exposure to carbon
88 nanomaterials was for less than 2 days, and cell viability [5] and total protein
89 concentration in bronchoalveolar lavage fluid had already been changed. Although it
90 may be said that these experiments evaluated the biological response of acute toxicity
91 by a proteomic approach, many of the expression-altered proteins in keratinocytes were

92 related to stress or the tox/detox response, and some of the proteins were altered by jet
93 fuel exposure [34]. Although jet fuel exposure caused acute and severe toxicity, it is not
94 the focus of our goal, which is to predict the long-term toxicity of CNTs.
95 The purpose of conventional proteomics is to detect alterations in protein expression
96 at the time of dynamic physiological phenomena or in the state of disease alteration [35].
97 On the other hand, few reports have examined the altered expression of proteins when
98 the external stimulus requires a long time to elicit a biological response. Such altered
99 protein expression would almost certainly occur if a biological response is predicted. We
100 analyzed protein alterations in cells exposed to carbon nanomaterials at concentrations
101 that either suppressed or did not alter cell proliferation which is the standard indicator
102 of the acute toxicity.
103 Human monoblastic leukemia cells (U937) were exposed to three grades of multi-wall
104 CNTs (MWCNTs), As-grown, HTT1800 and HTT2800 (Table 2), and carbon black (CB;
105 particle diameter = 85 nm) for 96 h [36, 37]. The iron and polycyclic aromatic
106 hydrocarbons in HTT1800 and HTT2800 were removed by thermally treating As-grown
107 MWCNTs at temperatures greater than 1800°C in argon. In our experiment, As-grown
108 MWCNTs exhibited significant inhibition of cell proliferation (n = 4). Therefore, we
109 thought that the As-grown MWCNTs produced a cytotoxic and/or cytostatic response (n

110 = 4). On the other hand, the proliferation of cells exposed to HTT1800 and HTT2800
111 tended to be inhibited, although this tendency was not statistically significant as
112 compared to the control ($n = 4$). HTT1800, with an amount of residual iron greater than
113 that of HTT2800, strongly inhibited the cell proliferation compared to HTT2800 [37].
114 CB did not affect cell proliferation at all [36]. Cell lysates were subjected to 2-DE and
115 the subsequent images were analyzed by PDquest software (Fig. 1). The proteins listed
116 in Table 3 were identified by peptide mass fingerprinting with matrix-assisted laser
117 desorption ionization time-of-flight mass spectrometry and had quantitatively
118 significant differences ($p < 0.05$) as compared to the control. The expression of many
119 proteins was altered in cells treated with HTT1800, HTT2800 or CB, a number of
120 proteins with altered expression were related to the degree of cell proliferation
121 inhibition. Altered expression of two proteins was shared by cells treated with any of the
122 three carbon materials. Expression alterations in these two proteins and an additional
123 12 were shared by cells treated with either HTT1800 or HTT2800. These proteins are
124 involved in: metabolic processes, signal transduction/cell communication, response to
125 stress, transport, cell differentiation, cell cycle and cell death. It is noteworthy that
126 there are proteins related to the response to stress or cell death that are altered without
127 the suppression of cell proliferation. However, the proteins that function in cell

proliferation and transcription were changed only in the case of cells exposed to MWCNTs with impurities. We can speculate on the farsighted cellular conditions from the current information available on function of the proteins that were altered by stimulation and the remarkable quantitative changes in these altered proteins. However, the function of these proteins is revealed at the time of remarkable alterations in their expression. Chronic toxicity attributed to slow alterations over time cannot be predicted due insufficient information on the relationship between chronic disease and alterations in protein expression. On the other hand, 22 proteins altered only in HTT1800 and six proteins altered only in HTT2800 and CB, respectively, may reflect the cellular response to fiber or particle properties or structural defects and impurities in each carbon nanomaterial. For example, annexin A2 is increased by MWCNTs but decreased by CB. Annexin A2 is modulated by TLR4, resulting in the secretion of inflammatory mediators [38]. The differences in the expression alteration of annexin A2 may be linked to cytokine production [19]. Alterations in cells that are divided two or three times while continually exposed to non-biodegradable CNTs, without exhibiting acute cytotoxic responses, may lead to CNT-induced chronic toxicity. Thus, we believe that the proteomic technique could be used to evaluate details of proteins related to CNT-induced chronic toxicity and clarify the pathophysiology of CNTs.

146

147 **Problem and direction of toxicoproteomics for carbon nanomaterials**

148 In this review, we do not speculate on the toxicity of CNTs based on the functions of
149 proteins with altered expression levels, because the scientific evidence on relationships
150 between the known functions of altered proteins and chronic toxicity is overwhelmingly
151 lacking. Chronic biological responses are highly influenced by environmental factors
152 and differences in individuals, whereas most of the acute toxic responses are
153 programmed with the conserved gene. Recently, clinical proteomic profiling to search for
154 biomarkers has been undertaken [39, 40], but the correlation coefficient to an individual
155 biomarker in a chronic disease is generally lower than that of an acute disease [41, 42].
156 This fact seems to be associated with the observation that higher organisms can adapt
157 to environmental alterations because they have multiple pathways for maintaining
158 homeostasis. For example, higher organisms may not develop a disease even if a
159 diagnostic biomarker is outside of its normal range. Therefore, in toxicoproteomic
160 research, only one or a few protein biomarkers are insufficient to assess long-term
161 toxicity. As a result, the hazard of useful compounds is overestimated; a wrong
162 conclusion may be drawn. To avoid such problems, all clinical and experimental
163 proteome data with quantitative information should be compiled into a database

without selecting specific proteins based on their degree of alteration. Multiple data from the same patients in different stages of a disease and different patients in the same disease stage are needed for successful applications of clinic proteomics, because the homeostasis and the state of a disease are kinetically altered [35, 43]. In other words, the key to the success of toxicoproteomic predictions of toxicity is the construction of a database of the detailed clinical proteome. Meanwhile, proteome data must be accumulated to investigate interspecies differences, individual differences and tissue differences using experimental animals and cultured cells with an ultimate goal of determining "personalized safety" from toxicoproteome data using novel models and tools, such as induced pluripotent stem cells derived from a specific individual.

In this review, we mainly introduced the proteomic approaches based on the 2-DE/MS strategy. Although the 2-DE/MS strategy can provide valuable information about protein profile changes associated with exposure to carbon nanomaterials, it may not allow for quantitative comparison of low abundant proteins. From this perspective, new quantitative proteomic approaches, such as isotope-labeled or label-free quantitative LC-MS/MS, also should be used to obtain more proteome information.

Conclusion

Based on the results of currently available toxicoproteomics, it is not yet clear if carbon nanomaterials will be hazardous in applications in various fields, including medical sciences. The most promising materials in the field of nanotechnology are carbon nanomaterials; therefore, their safety assessment should be performed very carefully. Carbon nanomaterials elicit different biological responses based on their shape, as seen from a comparison of MWCNTs and CB. As toxicoproteome data on the mechanisms of biological responses become available, the cytotoxicities of morphologically different carbon nanomaterials can be determined. The possibility of mesothelioma caused by CNTs is of particular importance; thus, a comprehensive safety assessment comprised of both toxicoproteomic analysis and other evaluation procedures should be performed. The properties of carbon nanomaterials, unlike the properties of asbestos, can be modified because the carbon nanomaterials are artificially produced. Therefore, if a hazard can be precisely identified, a new carbon nanomaterial without the hazardous property can be designed and produced. We believe that our lives will be enhanced by the development and medical application of nonhazardous carbon nanomaterials.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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362

363 **Figure Legends**

364 **Figure 1.** Proteome map [36, 37]. Numbered spots were changed by more than two-fold

365 with statistically significant differences ($p < 0.05$) in cells treated with HTT1800,

366 HTT2800 or CB as compared to the control cells ($n = 4$).

Table

Table 1. Summary of recent toxicological evaluation in carbon nanomaterials

in vivo

Type of carbon nanomaterials	Model	Methods	Summary results	Reference
MWCNT (Baytubes; Bayer MaterialScience) Φ; 10-16 nm	Wistar rats (inhalation exposure)	BAL analysis Gene expression analysis	Pulmonary inflammogenicity	[44]
MWCNT Φ; 20-50 nm, L; 5.9 , 0.7 μm (grinded, ungrinded, heated to 600℃, 2400℃; 2400℃ then grinded)	Wistar rats (i.t. instillation) rat lung epithelial cells	BAL analysis Micronucleus analysis	Inflammatory response Cytotoxicity Genotoxicity (lower effects with 2400℃ sample in comparison to 600℃ and unheated)	[45]
MWCNT (Nanocyl NC 7000; Nanocyl S.A.) Φ; 5-15 nm, L; 0.1-10 μm	Wistar rats (inhalation exposure)	BAL analysis Histopathology analysis Biomedical test	No systemic toxicity but multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis	[46]
MWCNT Φ; 11.3 nm, L; 0.7 μm	Wistar rats (i.p. administration)	Histopathology analysis	No mesothelioma No sustained inflammatory reaction	[13]
MWCNT (MWNT-7; Mitsui)	Fischer 344 rats (i.t. instillation)	Histopathology analysis	Intraperitoneally disseminated mesothelioma with bloody ascites	[47]
MWCNT (MWNT-7; Mitsui)	C57BL/6J mice (i.t. instillation)	BAL analysis Histopathology analysis	Pulmonary inflammation and damage Pulmonary fibrosis Granulomatous inflammation	[7]
MWCNT (MWNT-7; Mitsui) Φ; 100 nm, L; 10-20 μm C_{60} (Nanom purple; Frontier Carbon)	p53 (+/-) mice (i.p. administration)	Histopathology analysis	MWCNT induced mesothelioma C_{60} was no effect	[9]
MWCNT (Shenzhen Nanotech Port) Φ; 50 nm, L; 10 μm	Kunming mice (inhalation exposure)	BAL analysis Histopathology analysis	Pulmonary toxicity in 60-day	[48]
SWCNT (HiPco; Carbon Nanotechnologies) Φ; 0.8-1.2 nm, L; 0.1-1 μm	C57BL/6 mice (inhalation exposure) (i.t. instillation)	BAL analysis Histopathology analysis Collagen measurements Pulmonary function test K-*ras* mutation analysis	Inflammatory response Oxidative stress Collagen deposition Fibrosis Mutations of K-ras gene locus Inhalation > instillation	[8]
SWCNT (HiPco; Carbon Nanotechnologies) Φ; 0.8-1.2 nm, L; 0.1-1 μm SWCNT which was cut chemically Φ; 0.8-1.2 nm, L; 20-80 nm	Swiss mice (i.t. instillation) (i.p. administration)	Histopathology analysis Biomedical test	Neither death nor growth or behavioral troubles were observed. (oral) SWCNT (>10 μm) induced granuloma formation (i.p.)	[22]
Carbon black (Printex 90; Evonik Degussa) Φ; 14 nm Carbon black (Huber 990; H. Haeffner) Φ; 260 nm	Wistar rats (i.t. instillation)	BAL analysis	Lung inflammation and oxidant stress (Printex 90 > Huber 990)	[49]

in vitro

Type of carbon nanomaterials	Model used	Model methods	Summary results	Reference
MWCNT (XNRI WMVT-7; Bussan Nanotech Research) Φ; 67 nm, L; 1-13.5 μm	BEAS-2B human bronchial epithelial cells	Cell viability assay Cytokine assay	Cytotoxicity Inflammatory response	[1]
MWCNT (Graphistrength C100, ARKEMA) Φ; 12 nm, L; 0.1-12 μm Carbon black (FR101; Evonik Degussa) Φ; 95 nm	A549 human lung epithelial cells MeT5A human mesothelial cells	Cell viability assay Cell proliferation assay Apoptosis assay Oxidative stress assay	MWCNT; decrease in metabolic activity without changing cell membrane permeability or apoptosis Carbon black; any adverse effects	[14]
MWCNT Φ; 20 nm, L; 1.7 μm Carbon nanofibers (Pyrograf Products) Φ; 150 nm, L; 5 μm Carbon black (obtained after grinding graphite) submicrometer	Human lung tumor cells • Calu-1 epidermoid carcinoma lung • H446 small cell carcinoma • H596 Adenosquamous carcinoma	Cell viability assay	Cytotoxicity; Carbon black>Carbon nanofibers>MWCNT	[50]
MWCNT (Shenzhen Nanotech Port) Φ; 10-20 nm, L; 0.5-40 μm SWCNT Φ; 1.4 nm, L; 1 μm C_{60}	Alveolar macrophage form guinea pig	Cell viability assay Phagocytic ability	Cytotoxicity; SWCNT>MWCNT>C_{60} SWCNT significantly impaired phagocytosis.	[51]
MWCNT (Nanostructured & Amorphous Materials) Φ; 10-20, 30-50 nm SWCNT (Nanostructured & Amorphous Materials) Φ; 1-2 nm Carbon black (Printex 90; Evonik Degussa) Φ; 14 nm	NR8383 rat alveolar macrophage cells A549 human lung epithelial cells	Cell viability assay ROS analysis Cytokine assay NO assay	No sign of acute toxicity	[4]
SWCNT (HiPco; Carbon Nanotechnologies) Φ; 0.8-1.2 nm, L; 0.1-1 μm	A549 human lung epithelial cells	Cell viability assay Clonogenic assay	Cytotoxicity (reduction of proliferative capacity)	[52]
SWCNT (HiPco; Carbon Nanotechnologies)	A549 human lung epithelial cells Normal human primary bronchial epithelial cells	Cell viability assay Cytokine assay	Low cytotoxicity Suppression of inflammatory responses	[53]
SWCNT (EliCarb; Thomas Swan) Φ; 0.9-1.7 nm, L; <1 μm C_{60} (Sigma-Aldrich) Φ; 0.7 nm Carbon black (Printex 90; Evonik Degussa) Φ; 14 nm	FE1Muta™ mouse lung epithelial cells	LDH assay Cell cycle analysis ROS analysis Comet assay Mutagenicity analysis Mutation analysis	No cytotoxicity SWCNT slowed cell proliferation. Carbon black produced ROS most and induced mutation. SWCNT and C_{60} were less genotoxic.	[2]
SWCNT (National Institute of Standards and Technology) Φ; 1.4 nm, L; 2-5 μm	Normal & malignant human mesothelial cells	Cell viability assay ROS analysis Comet assay	ROS generation Cell death DNA damage	[3]
SWCNT + CNT (Sigma-Aldrich) Φ; 1.1 nm, L; 0.5-100 μm Graphite nanofibres (Sigma-Aldrich) Outer Φ; 80-200 nm, L; 5-20 μm	BEAS-2B human bronchial epithelial cells	Cell viability assay Comet assay Micronucleus assay	Cytotoxicity Genotoxicity • Comet assay; dose-dependent • Micronucleus assay; Not dose-dependent	[54]
Carbon black (Printex 90; Evonik Degussa) Φ; 14 nm Carbon black (Huber 990; H. Haeffner) Φ; 260 nm	A549 human lung epithelial cells	LDH assay Comet assay Cell cycle analysis	Printex 90 induced DNA damage and altered cell cycle kinetics.	[55]

Φ; diameter, L; length, C_{60}; fullerene, i.t.; intratracheal, i.p.; intraperitoneal, BAL; bronchoalveolar lavage, ROS; reactive oxygen species, LDH; lactate dehydrogenase

Table

Table 2. Basic properties of multi-walled carbon nanotubes [37]

	As-grown	HTT1800	HTT2800	Testing method
Diameter (nm)	100-150	100-150	100-150	FE-SEM
Length (μm)	10-20	10-20	10-20	FE-SEM
d_{002} (Å)	-	-	0.339	X-ray diffraction
R value (I_d/I)[a]	1.041	0.855	0.051	Raman spectroscopy
Specific surface area (m^2/g)	-	26	13	N_2 adsorption
Real density (g/cm^3)	-	-	2.09	Pycnometer
Iron content (ppm)	12,000	80	<20	ICP-MS
Soluble iron content (%)[b]	0	91	100	ICP-MS
Polycyclic aromatic hydrocarbons (wt%)[c]	0.19	None	None	GC-MS
Oxidation temperature (^oC)[d]	630	720	820	TGA

a R refers to the intensity of D band over the intensity of G band.

b We have determined the dissolved amount of iron by refluxing 5 g of nanotubes in hydrochloric acid (0.6 N) for 25 h.

c We have measured acetone-soluble components.

d We have determined the oxidation temperatures via the derivation of TGA curve.

FE-SEM; Field emission-scanning electron microscopy, ICP-MS; Inductively coupled plasma-mass spectrometry, GC-MS; Gas chromatograph-mass spectrometry, TGA; Thermogravimetric analysis

Table

Table 3 Identified proteins [36,37]

Spot No.	Protein Name	Theoritical MW	Theoritical pI	MOWSE Score	Coverage	Matched Peak	Ratio HTT 1800	Ratio HTT 2800	Ratio Carbon black
1	heterogeneous nuclear ribonucleoprotein A2/B1	37478	8.97	141	43%	14/18	* 2.12	# 1.45	1.08
2	DnaJ homolog subfamily C member 8	29823	9.04	89	28%	10/12	1.00	0.74	* 0.42
3	small nuclear ribonucleoprotein polypeptide A'	28540	8.72	97	29%	8/11	* 0.23	0.55	0.30
4	proteasome subunit β type-1	26757	8.27	82	27%	7/8	* 0.36	# 0.59	0.76
5	annexin A2	38864	7.57	114	28%	11/12	* 3.58	2.50	* 0.34
6	vasodilator-stimulated phosphoprotein	39977	9.05	78	24%	8/15	* 3.20	1.58	3.21
7	heterogeneous nuclear ribonucleoprotein M	77819	8.84	186	32%	24/26	* 2.01	1.25	1.23
8	phosphatidylethanolamine-binding protein 1	21186	7.01	107	45%	8/13	* 0.35	* 0.44	0.87
9	flavin reductase	22248	7.13	74	40%	6/9	* 0.13	0.42	0.68
10	pyruvate kinase isozymes M1/M2	58664	7.60	129	16%	14/14	* 2.62	1.29	1.82
11	transketolase	68687	7.58	242	30%	23/25	* 2.22	1.84	1.37
12	proteasome subunit α type-2	26024	6.92	76	17%	6/9	* 0.47	0.56	0.69
13	triosephosphate isomerase	27008	6.45	134	42%	11/15	* 0.43	* 0.45	0.77
14	phosphoglycerate mutase 1	28928	6.67	84	20%	6/6	* 0.49	# 0.51	# 0.71
15	actin related protein 2/3 complex subunit 2	34454	6.84	72	19%	8/16	0.88	# 0.60	* 0.49
16	actin related protein 2/3 complex subunit 2	34454	6.84	72	19%	7/10	0.59	* 0.50	1.22
17	6-phosphogluconate dehydrogenase, decarboxylating	53745	6.80	71	12%	6/6	0.58	* 0.49	* 0.47
18	far upstream element-binding protein 2	73542	6.84	140	22%	12/13	* 2.35	2.06	1.20
19	cytosolic malate dehydrogenase	36687	6.91	87	20%	9/11	* 0.49	# 0.65	0.84
20	lamin A/C	65167	6.40	193	34%	19/19	1.73	* 2.47	0.84
21	mitochondrial import receptor subunit TOM70	68264	6.75	69	12%	7/8	* 2.34	1.81	0.87
22	polyribonucleotide nucleotidyltransferase 1	86664	7.87	86	13%	12/18	* 2.32	1.82	0.88
23	δ-1-pyrroline-5-carboxylate synthetase	88171	6.66	87	9%	8/8	* 2.10	# 1.79	1.50
24	transaldolase	37730	6.36	167	36%	16/18	* 0.45	0.58	0.69
25	squalene synthetase	48724	6.10	68	17%	8/12	* 0.47	0.65	0.98
26	α-ketoglutarate dehydrogenase	117353	6.40	119	12%	14/15	* 4.13	* 4.10	1.02
27	protein DJ-1	20092	6.33	114	43%	12/13	* 0.42	* 0.35	0.89
28	heat shock protein β-1	22840	5.98	90	26%	8/9	# 0.59	* 0.42	0.92
29	transaldolase 1	37730	6.36	150	29%	14/15	* 0.35	0.46	0.66
30	serine/threonine-protein phosphatase PP1-α catalytic subunit	38411	5.94	120	34%	11/18	# 0.52	* 0.29	0.92
31	leukocyte elastase inhibitor	42857	5.90	133	32%	13/14	0.65	* 0.48	0.99
32	serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform	52299	5.82	103	16%	9/10	0.72	* 0.49	1.20
33	interferon-induced protein 53	49247	6.03	76	10%	6/6	* 0.43	0.80	0.78
34	DNA mismatch repair protein Msh2	105600	5.58	69	10%	9/12	* 3.13	* 3.78	1.97
35	neutral α-glucosidase AB	107375	5.74	183	19%	21/24	* 3.09	* 3.05	2.15
36	F-actin capping protein subunit β	31036	5.69	104	26%	10/13	* 0.36	0.42	1.34
37	Thioredoxin domain-containing protein 5	44636	5.77	68	12%	6/8	1.21	1.04	* 0.20
38	heat shock protein 60	61229	5.70	172	28%	15/15	* 2.70	1.43	1.70
39	lamin-B2	67790	5.29	183	31%	19/19	* 2.44	1.71	0.99
40	14-3-3 protein γ	28498	4.80	150	36%	16/19	# 0.50	* 0.48	* 0.39
41	elongation factor 1-δ	31245	4.90	124	34%	8/8	0.33	* 0.18	0.53
42	Ubiquitin thioesterase OTUB1	31549	4.85	81	26%	6/8	0.48	0.23	* 0.23
43	Spermine synthase	24942	5.16	71	19%	6/10	0.68	0.67	* 0.34
44	splicing factor 3A subunit 3	59238	5.27	76	14%	9/10	1.60	1.52	* 0.46
45	78 kDa glucose-regulated protein	72431	5.07	176	27%	19/20	* 2.33	1.27	0.73
46	transportin 1	103091	4.81	102	13%	12/15	1.47	4.35	* 3.80
47	DNA damage-binding protein 1	128470	5.16	84	7%	10/11	2.10	* 2.82	1.82
48	14-3-3 protein ε	29369	4.63	150	43%	17/21	* 0.41	0.59	0.89
49	proliferating cell nuclear antigen	29177	4.57	115	28%	12/15	* 0.47	0.63	0.78
50	splicing factor SC35	25461	11.86	100	38%	10/12	* 0.27	0.30	1.02
51	ribonuclease inhibitor	52214	4.71	68	15%	6/8	* 0.44	0.61	0.61
52	calreticulin	48325	4.29	68	13%	6/8	* 4.88	1.52	12.02

Ratios of protein expression were compared to the control. n =4. *; p <0.05 and two-fold change, #; p <0.05 only

Figure

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Figure 1