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# Nuclear proteome analysis of cisplatin-treated HeLa cells

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### ABSTRACT

Cisplatin has been widely accepted as one of the most efficient anticancer drugs for decades. However, the mechanisms for the cytotoxic effects of cisplatin are still not fully understood. Cisplatin primarily targets DNA, resulting in the formation of DNA double strand breaks and eventually causing cell death. In this study, we applied two-dimensional electrophoresis coupled with LC-MS/MS to analyze the nuclear proteome of HeLa cells treated with cisplatin, in an effort to uncover new mechanistic clues regarding the cellular response to cisplatin. A total of 19 proteins were successfully identified, and these proteins are involved in a variety of basal metabolic and biological processes in cells, including biosynthesis, cell cycle, glycolysis and apoptosis. Six were related to the regulation of mRNA splicing, and we therefore asked whether the *Fas* gene might undergo alternative splicing following cisplatin treatment. This proved to be the case, as the splicing forms of *Fas* were modified in cisplatin-treated HeLa cells. This work provides novel information, from the perspective of the nuclear response, for understanding the cytotoxicity caused by cisplatin-induced DNA damage.

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## 1. Introduction

Cisplatin (*cis*-diamino-dichloro-platinum(II), CDDP) is a platinum-based anticancer drug. During the 30 years since its introduction [1], cisplatin has been widely used in the chemotherapeutic treatment of various cancers, including ovarian, testicular, bladder and lung cancers. Despite the marked effects of cisplatin, the underlying mechanisms have not yet been determined. It is generally accepted that DNA is the most important target of cisplatin [2]. Cisplatin can form a variety of DNA adducts, including intrastrand crosslinks, interstrand crosslinks, monoadducts and DNA–protein crosslinks [3,4]. In dividing cells, a replication fork can be stalled at the sites of DNA adducts, resulting in the formation of DNA double strand breaks (DSBs), which are regarded as the most severe type of DNA damage [5]. By altering the dynamic and structural properties of DNA, DSBs modulate many aspects of DNA metabolism, including DNA replication, recombination, transcription, and repair. DSBs can also activate several signal transduction pathways that eventually lead to cell malfunctioning and cell death [6–9].

The histone variant H2AX has moved to the center of research attention regarding the cellular responses to DSBs since the discovery that this protein becomes phosphorylated at Ser-139 (termed  $\gamma$ H2AX) by members of the phosphatidylinositol 3-kinase (PI3K) family in the vicinity of DSBs [10,11].  $\gamma$ H2AX is also required to recruit a number of DSB response proteins including repair factors and chromatin remodeling complexes [12–14]. Recently,  $\gamma$ H2AX foci have become recognized as an effective indicator of DSBs, applicable even under conditions where only a few DSBs are elicited [15,16]. Thus, by visualising  $\gamma$ H2AX foci formation, it is possible to estimate the frequency of DSB formation and to tie these events to changes in the protein expression profiles.

Despite these recent advances in our understanding, the mechanisms by which cisplatin-induced DNA damage leads to its associated cytotoxic effects are not yet clearly defined. The large-scale screening approaches enabled by the emergence of the field of proteomics has provided researchers with novel possibilities for systematically approaching biological questions that would be impossible to address using classical biology techniques [17]. Recently, several studies have employed proteomic methods to address questions related to the mechanism(s) of cisplatin-associated toxicities. From these studies, a number of proteins possibly related to the mechanism of cisplatin-induced damage or cisplatin resistance were identified. The functions of these proteins are related to a variety of basal metabolic and biological processes in cells, including biosynthesis, cell cycle, glycolysis and apoptosis

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[18–20]. However, no underlying control mechanism or pathway has yet been clearly identified. Since DNA is the primary target of cisplatin damage and because the cellular response to DNA damage occurs mainly in the nucleus, we wished to investigate the effects of cisplatin-induced DNA damage on nuclear protein expression profiles, as this approach seemed likely to provide novel information that could help to elucidate the molecular mechanisms and pathways underlying the cytotoxicity of cisplatin. In the present study, therefore, the effect of cisplatin treatment on protein expression in human cervical adenocarcinoma HeLa cells was examined, using two-dimensional gel electrophoresis (2DE) coupled with liquid chromatography tandem mass spectroscopy (LC–MS/MS) technique. We found that proteins affected were involved in a number of cellular processes such as pre-mRNA splicing, stress response, cell cycle progression and mitosis. This work therefore has provided novel and important insights, from the perspective of the nuclear response, into the mechanisms of cytotoxicity caused by cisplatin-induced DNA damage.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Cisplatin (*cis*-Diammineplatinum(II) dichloride) was purchased from Sigma–Aldrich (Saint Louis, USA). Antibodies for immunofluorescence microscopy and immunoblotting were obtained from the following sources: Lamin A/C (Biovision, Mountain View, CA, USA), BUB3 (BD Biosciences, San Diego, CA, USA), and  $\gamma$ H2AX (Upstate Biotechnology, Lake Placid, NY, USA). An affinity-purified peptide antibody against PSP1 was generated in rabbit by Fox et al. [21]. FITC-conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from Zhongshan Biotechnology (Beijing, China).  $\beta$ -Actin and the HRP-conjugated secondary antibodies were from Multisciences Biotechnology (Hangzhou, China).

### 2.2. Cell culture and treatment

Human cervical adenocarcinoma HeLa cells were cultured in Eagle's Minimum Essential Medium (MEM; Gibco, Carlsbad, CA) supplemented with 10% newborn calf serum (PAA, Linz, Austria) and 1% penicillin/streptomycin (100 U/ml and 100  $\mu$ g/ml; PAA). HeLa cells were exposed to 0.1, 1, 5 or 10  $\mu$ M cisplatin and prepared as described for the individual experiments. Cells treated with 0.9% sodium chloride were used as the control group.

### 2.3. Cytotoxicity assay

HeLa cells were seeded into a 96-well culture plate, and then after a 24 h incubation, exposed to cisplatin treatment for 12 h. The cytotoxic effect of cisplatin was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [22]. In order to dissolve the MTT formazan crystals and to avoid interference due to the presence of phenol red, the medium was replaced with 150  $\mu$ l isopropanol. Absorbance at 570 nm was determined on a multimode reader (Infinite 200, Tecan, Austria).

### 2.4. Protein extraction and 2D electrophoresis (2DE)

Nuclear proteins were extracted from cells treated or untreated with cisplatin using the Nuclear–Cytosol Extraction Kit (Applygen Technologies Inc., Beijing, China). Extracts were dialyzed against several changes of distilled water, due to the sensitivity of isoelectric focusing to salt concentration. Protein concentration was measured using the Bradford assay [23]. The resulting supernatants were stored in aliquots at  $-70^{\circ}\text{C}$  until further analysis.

For 2DE analysis, the nuclear extracts were applied on 17-cm, pH 5–8 linear immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA, USA) for isoelectric focusing and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Detailed conditions and steps were taken as established previously in our laboratory [24]. The gels were then removed and stained by a silver-staining method described before [25].

### 2.5. Image analysis and in-gel digestion

Image analysis and in-gel digestion was conducted following the protocol as previously described [24].

### 2.6. LC–MS/MS

Tryptic digests were introduced from an autosampler (CTC Analytics, Bern, Switzerland) via a peptide trap (Michrom BioResources, Auburn, CA) onto a 5  $\mu$ m

100A Magic C18 analytical column (Michrom Bioresources) at a flow of 1  $\mu$ l/min. A linear gradient was delivered from 5 to 35% acetonitrile for 30 min to fractionate tryptic peptides. The eluate was introduced directly onto a QSTAR Elite mass spectrometer (Applied Biosystems SCIEX, Concord, ON, Canada) via a 10- $\mu$ m emitter tip (PicoTip; New Objective, Woburn, MA, USA). Typically, a voltage between 1800 and 2400 V was applied to the liquid stream to induce ionization. One-second MS (mass spectroscopy) scans were used to identify candidates for fragmentation during MS/MS scans. Up to four 2-s MS/MS scans were collected after each scan. All protein identifications were performed with ProteinPilot™ 2.0 software (Applied Biosystems). The spectra were searched with 0.15-Da mass tolerance in the NCBI nr or Swiss-Prot nr database (from April 23, 2008; <ftp://ftp.ncbi.nih.gov/blast/db/FAST/nr.gz>) [26].

### 2.7. Immunofluorescence microscopy

HeLa cells were seeded into a 6-well culture plate containing a 22-mm-diameter coverslip in each well, and immunofluorescence microscopy was conducted as described previously [24]. Finally, the coverslips were mounted onto microscope slides in 90% glycerol and observed with a Leica DMI 4000 immunofluorescent microscope.

### 2.8. Immunoblotting

Cells were lysed in RIPA lysis buffer (Beyotime, Nantong, China), and protein concentrations were determined using BCA (bicinchoninic acid) Protein Assay Kit (Beyotime). Denatured protein extracts were loaded and separated on 10% SDS–polyacrylamide gels (Mini-Protein II, Bio-Rad) and transferred to an Immoblot PVDF (polyvinylidene fluoride) Membrane (Bio-Rad). After blocking with 5% non-fat milk in Tris–buffered saline with 0.1% (v/v) Tween-20 (TBST), membranes were incubated with primary antibodies at  $4^{\circ}\text{C}$  overnight, followed by incubation of HRP-conjugated secondary antibodies for 1 h. After three washes, membrane-bound proteins of interest were detected using the EZ-ECL Chemiluminescence Detection Kit (Kibbutz Beit Haemek, Israel) and then exposed to X-ray films.

### 2.9. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 5  $\mu$ g of total RNA was used for first-strand cDNA synthesis with SuperScript III (Invitrogen) and random hexamer (Sangon, Shanghai, China). The cDNA obtained was normalized by reverse transcription polymerase chain reaction (RT-PCR). PCR was performed at  $94^{\circ}\text{C}$  for 30 s, at  $57^{\circ}\text{C}$  for 30 s, and at  $72^{\circ}\text{C}$  for 1 min for 35 cycles using TaKaRa Taq™ (TaKaRa, Otsu, Japan). For the analysis of *Fas* isoforms expression pattern, primers were 5'-GACATGGCTTAGAAGTGGAAA-3' and 5'-TTAGTGTCTGACTCCAGCAA-3' [27].

### 2.10. DNA-PAGE assay

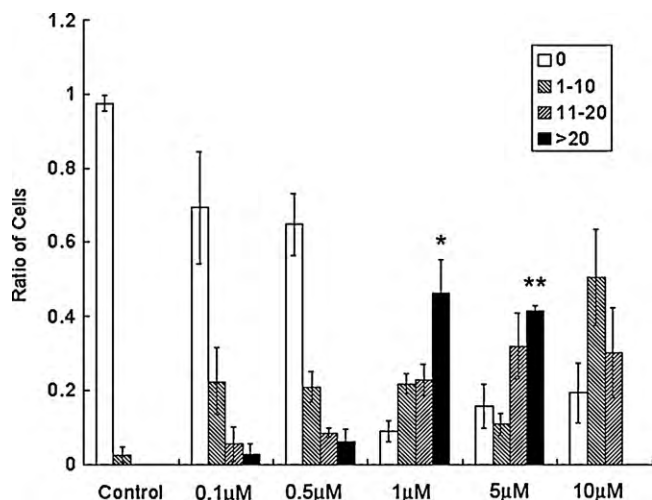
The RT-PCR products were loaded on 8% urea-polyacrylamide gel and separated at 100 V for 4 h. The gel was then stained in 0.1% silver nitrate solution and sodium hydroxide solution (1.5% sodium hydroxide, 0.019% anhydrous borax and 0.4% formaldehyde) until DNA band appeared. Finally, the gel was settled in 10% acetic acid for further image collection.

## 3. Results

### 3.1. Cisplatin treatment induces cell death and the formation of $\gamma$ H2AX foci in HeLa cells

The effect of cisplatin on the viability of HeLa cells was first examined by treating cells with 0.1, 0.5, 1, 5, 10, 20, 30, 50, 100  $\mu$ M cisplatin for 12 h, then performing the MTT test. Cisplatin did not cause obvious cell death within this time frame until its concentration reached 10  $\mu$ M, and the survival rate decreased steadily as the treatment concentration was raised beyond this point (data not shown).

$\gamma$ H2AX focus formation is considered to serve as an effective marker of DSBs, and it has been reported that the formation of these foci could be induced by cisplatin at different exposure intervals [28]. Here, we detected  $\gamma$ H2AX focus formation by immunofluorescence microscopy in HeLa cells after treatment with 0.1, 0.5, 1, 5 and 10  $\mu$ M cisplatin for 12 h. The quantitative data shown in Fig. 1 demonstrate that cisplatin at low concentrations can induce  $\gamma$ H2AX foci formation modestly. When treated with 1  $\mu$ M cisplatin, for example, about half of the cells displayed more than 20 foci/cell and cells with no foci accounted only 10% of the total. The number of foci increased further when the concentration of cisplatin

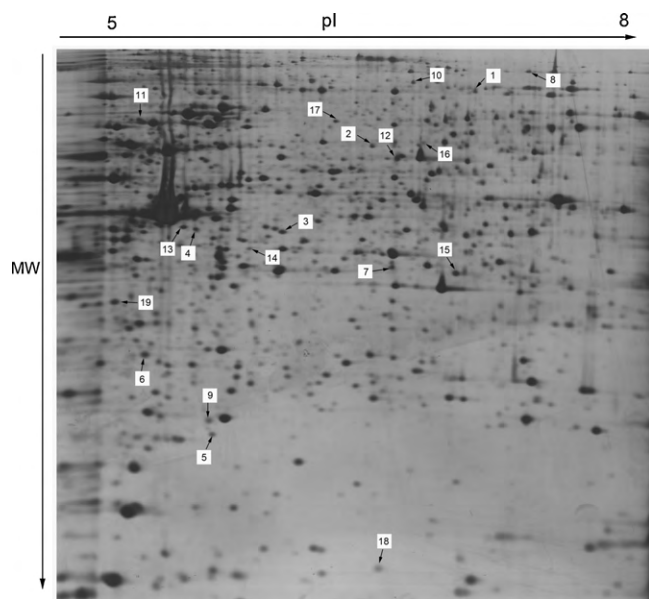


**Fig. 1.**  $\gamma$ H2AX foci formation induced by cisplatin treatment. HeLa cells were treated with 0.1, 0.5, 1, 5 and 10  $\mu$ M cisplatin for 12 h. Treatment with 0.9% NaCl was used as the control. Cells with different numbers of foci were divided into four groups, and the ratio of each group was calculated. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to control.

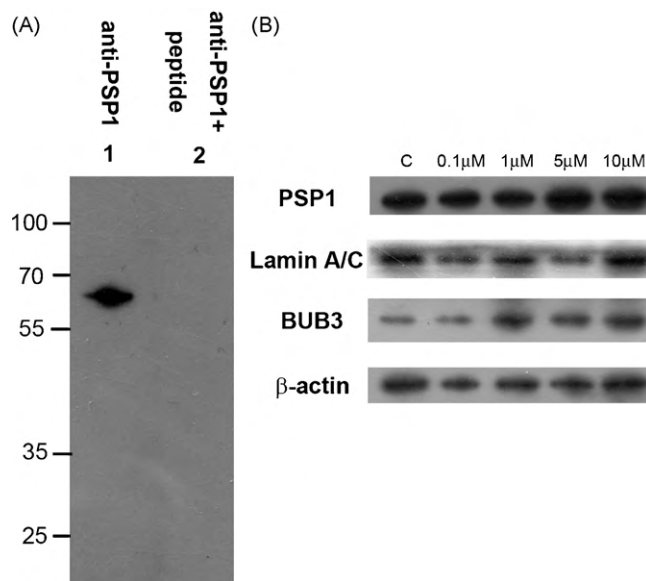
was increased to 5  $\mu$ M. However, when the cisplatin concentration reached 10  $\mu$ M, the number of  $\gamma$ H2AX foci decreased, as cells with more than 20 foci/cell were not detected, possibly due to cell death (Fig. 1). Based on the cytotoxicity and genotoxicity results observed, treatment concentrations of 0.1, 1, 5 and 10  $\mu$ M were chosen for the nuclear proteomic study in HeLa cells.

### 3.2. Cisplatin treatment alters the nuclear protein expression profile in HeLa cells

Nuclear proteins from HeLa cells, treated or untreated with 0.1, 1, 5 and 10  $\mu$ M cisplatin for 12 h, were separated by isoelectric focusing and SDS-PAGE. For each pair of treatment and control samples, three pairs of gels were analyzed. A representative silver-stained image of the nuclear protein expression profile in HeLa cells is shown in Fig. 2. After detection of spots, background subtraction



**Fig. 2.** Representative 2-DE pattern of nuclear extracts from HeLa cells. Proteins were focused on 17 cm, pH 5–8 linear strips. Black arrows indicate the spots identified using LC-MS/MS after silver staining and in-gel digestion. Details of the corresponding spots are listed in Table 1.



**Fig. 3.** Immunodetection of PSP1, Lamin A/C and BUB3 by Western blot. (A) PSP1 protein in HeLa extract. The blot of anti-PSP1 was combined with that of anti-PSP1 preincubated with an excess of cognate peptide. The major band of ~60 kDa in size was detected specifically by the antibody against PSP1. (B) Detection of PSP1, Lamin A/C and BUB3 protein expression in control and cisplatin treatment groups.  $\beta$ -actin was used as control. Immunoblotting was detected by chemiluminescence followed by exposure to X-ray films.

and volume normalization, the number of protein spots in each group were as follows: 749  $\pm$  23 in the 0.1  $\mu$ M cisplatin treatment group and 749  $\pm$  31 in the corresponding control group; 769  $\pm$  22 in the 1  $\mu$ M cisplatin treatment group and 732  $\pm$  20 in the control group; 654  $\pm$  32 in the 5  $\mu$ M cisplatin treatment group and 604  $\pm$  2 in the control group; 763  $\pm$  11 in the 10  $\mu$ M cisplatin treatment group and 745  $\pm$  33 in the control group. Compared to the control groups, no protein spot appeared nor disappeared in cisplatin-treated HeLa cell nuclei. However, the relative expression levels for some proteins did change. A total of 35 spots were detected where the expression level was altered by cisplatin treatment by a factor of at least twofold, of which 14 spots were induced by cisplatin and the other 21 were suppressed.

The 35 protein spots which were expressed differentially after cisplatin treatment were excised from their corresponding gels, in-gel digested and subjected to LC-MS/MS analysis. Ultimately, a total of 19 spots were identified by NCBI nr or Swiss-Prot nr database searching (Fig. 2 and Table 1). These proteins have been shown to be involved in various aspects of basal metabolism and biological process in cells. We were unable to identify the remainder of the 35 protein spots, due either to insufficient signal or to unsuccessful MS analysis.

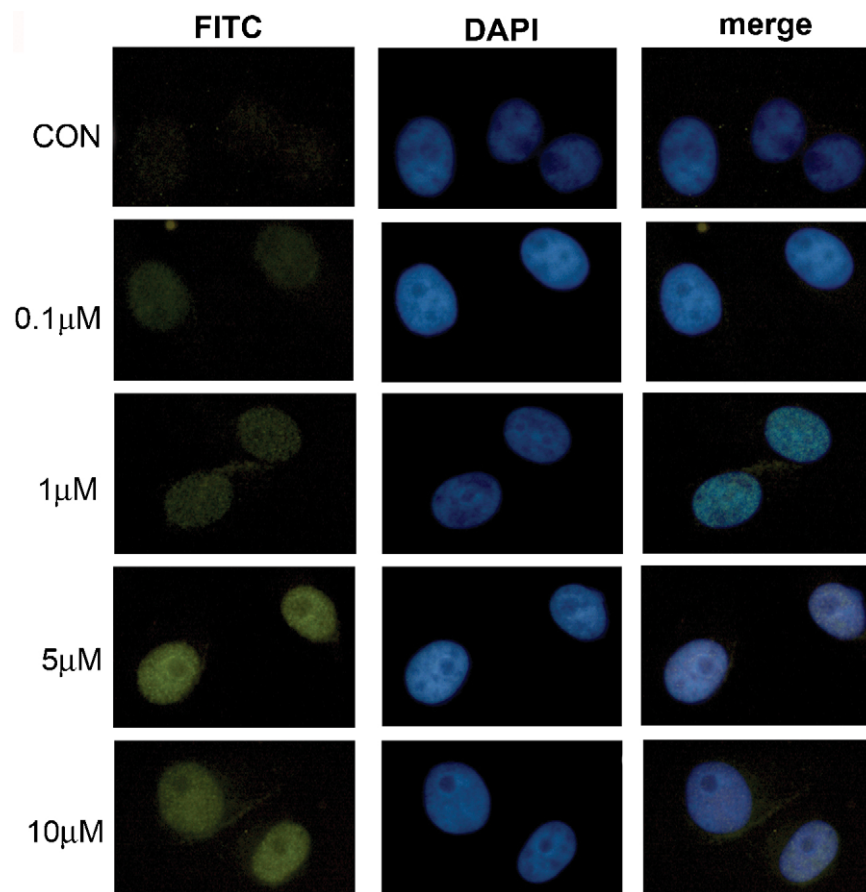
### 3.3. Confirmation of differential protein expression in cisplatin treated or untreated HeLa cells

To confirm the results obtained by 2DE analysis, we estimated by immunoblot the expression of paraspeckle component 1 (PSP1), Lamin A/C and BUB3 in HeLa cells treated or untreated with different concentrations of cisplatin, as previous studies indicated their possible involvement in DNA damage response [28–31]. The antibody against PSP1 was generated according to Fox et al. [21], and results from immunoblot verified its specificity to PSP1 protein (Fig. 3A). The results showed that the expression level of PSP1 was induced by 5  $\mu$ M cisplatin and that the level of BUB3 was induced in all treatment groups, while the expression level of Lamin A/C was down-regulated by treatment with 5  $\mu$ M cisplatin (Fig. 3B).

**Table 1**  
Nuclear proteins in HeLa cells induced or repressed after different concentrations of cisplatin treatments.

Spot	Symbol	Identification	Swiss-Prot accession no.	Theoretical pI and Mr	Function	<sup>a</sup> Ratio of spot volume (P-value)			
						0.1 μM	1 μM	5 μM	10 μM
1	<b>KSRP</b>	<b>Far upstream element-binding protein 2</b>	<b>Q92945</b>	<b>6.84/73 146.43</b>	<b>mRNA trafficking and processing</b>	<b>3.0299 (0.0045)</b>			
2	<b>PSP1</b>	<b>Paraspeckle component 1</b>	<b>Q8WXF1</b>	<b>6.26/58 743.56</b>	<b>Transcription regulation</b>	<b>2.2440 (0.0258)</b>			
3	<b>hnRNP H</b>	<b>Heterogeneous nuclear ribonucleoprotein H</b>	<b>P31943</b>	<b>5.89/49 229.47</b>	<b>Pre-mRNA processing</b>	<b>2.2741 (0.0152)</b>			
4	RUVBL2	RuvB-like 2	Q9Y230	5.49/51 156.57	Transcription regulation	2.5660 (0.0184)			
5	CDK4I	Cyclin-dependent kinase 4 inhibitor A	P42771	5.52/16 532.64	Negative regulation of cell proliferation	2.6874 (0.0076)			
6	RanBP1	Ran-binding protein 1	P43487	5.19/23 310.12	Signal transduction	2.1617 (0.0148)			
7	BUB3	Mitotic checkpoint protein BUB3	O43684	6.36/37 154.78	Mitosis-related	2.1922 (0.0198)			
8	C4orf23	Probable tRNA (uracil-O(2)-)-methyltransferase	Q8IYL2	6.98/84 628.90	Methyltransferase activity	2.3221 (0.0233) 2.0917 (0.0502)			
9	NDK A	Nucleoside diphosphate kinase A	P15531	5.83/17 148.73	Synthesis of nucleoside triphosphates	2.1247 (0.0062)			
10	LMNA	Lamin-A/C	P02545	6.57/74 139.49	Components of nuclear lamina	0.4313 (0.0285) 0.4838 (0.0118)			
11	LMNB	Lamin-B1	P20700	5.11/66 408.34	Components of nuclear lamina	0.4572 (0.0325)			
12	3-PGDH	D-3-phosphoglycerate dehydrogenase	O43175	6.29/56 650.50	Amino-acid biosynthesis	0.4708 (0.0014)			
13	<b>DDX39</b>	<b>ATP-dependent RNA helicase DDX39</b>	<b>O00148</b>	<b>5.46/49 129.51</b>	<b>Pre-mRNA processing</b>	<b>0.4646 (0.0408)</b>			
14	<b>SPF45</b>	<b>Splicing factor 45</b>	<b>Q96I25</b>	<b>5.76/44 961.54</b>	<b>Pre-mRNA processing</b>	<b>0.3370 (0.0360)</b>			
15	RFC5	Replication factor C subunit 5	P40937	6.72/38 496.58	DNA replication	0.2978 (0.0112)			
16	TyrRS	Tyrosyl-tRNA synthetase	P54577	6.61/59 143.48	Tyrosyl-tRNA aminoacylation	0.3777 (0.0047)			
17	MSN	Moesin	P26038	6.08/67 820.04	Signal transduction	0.4555 (0.0098)			
18	Ub	Ubiquitin	P62988	6.56/8 564.84	Protein ubiquitination	0.1988 (0.0424)			
19	<b>hnRNP C</b>	<b>Heterogeneous nuclear ribonucleoprotein C</b>	<b>Q5RA82</b>	<b>4.95/33670.01</b>	<b>Pre-mRNA processing</b>	<b>0.4004 (0.0084)</b>			

<sup>a</sup> Ratio of spot volume means the ratio of relative spot volume of treatment group to control group. Proteins related to mRNA splicing are bolded in the table.



**Fig. 4.** Cisplatin induces BUB3 foci formation. After 12 h treatment of 0.1, 1, 5 or 10  $\mu\text{M}$  cisplatin, cells were fixed and stained. Cells treated with 0.9% sodium chloride instead were used as control. The BUB3 foci were labeled with FITC, and the nuclei were stained with DAPI.

These results are consistent with those obtained through the 2-DE analysis, thus verifying the results of our proteomic study.

Immunofluorescence microscopy was then performed in order to detect differences in expression and subcellular localization of BUB3 and PSP1 in HeLa cells treated with cisplatin. The results demonstrated that BUB3 could be slightly induced by 0.1  $\mu\text{M}$  cisplatin, and was significantly induced when cells were treated with 5  $\mu\text{M}$  cisplatin (Fig. 4). The immunofluorescent images also indicated that BUB3 localizes mainly in the nuclei.

Unlike BUB3, PSP1 was found constitutively expressed in HeLa cell nuclei and was not significantly induced by low levels of cisplatin. However, in the 5  $\mu\text{M}$  treatment group, we observed a significant increase in the PSP1 expression level, although this increase was attenuated in cells treated with 10  $\mu\text{M}$  cisplatin (Fig. 5). In these same cells, we also examined  $\gamma\text{H2AX}$  foci expression by staining with FITC-labeled antibody to see if PSP1 colocalized with  $\gamma\text{H2AX}$  foci after DNA was damaged by cisplatin. To address this question, we merged the images of  $\gamma\text{H2AX}$  foci and PSP1 in HeLa cells, and found that most of the PSP1 foci did not colocalize with  $\gamma\text{H2AX}$  foci, in either the presence or absence of cisplatin treatment. Together, these results confirm the differential expression of BUB3 and PSP1 in cisplatin-treated HeLa cells, as well as their predominant nuclear localization.

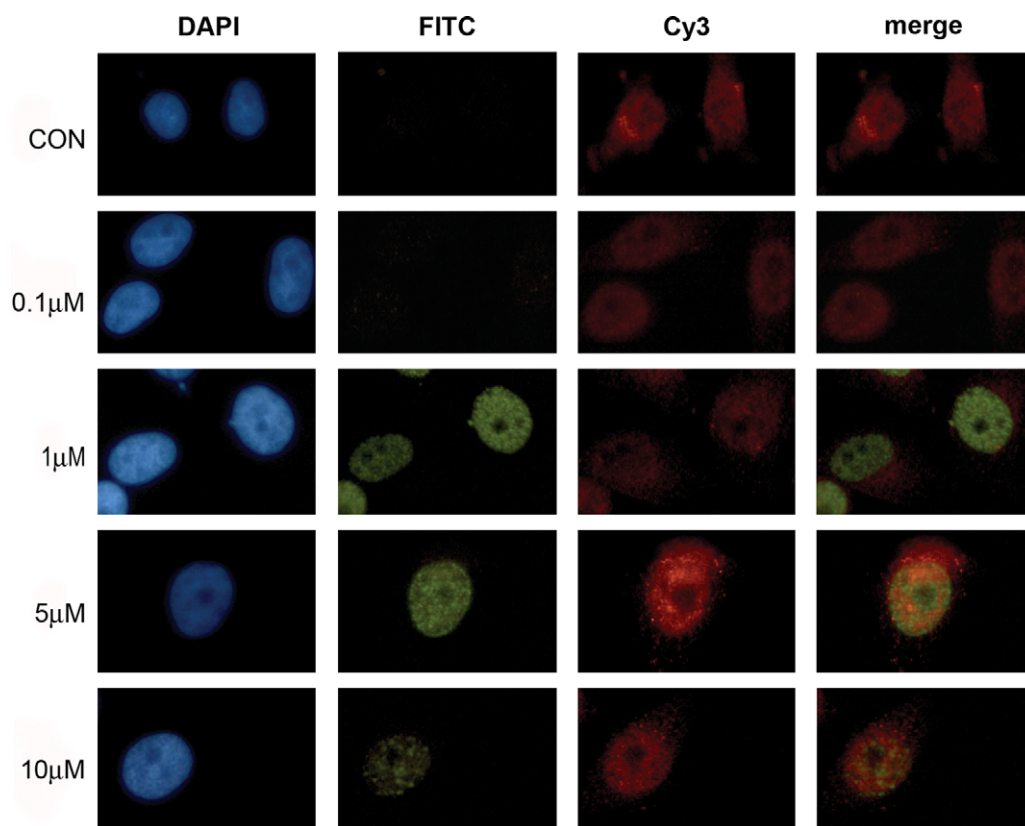
#### 3.4. Cisplatin treatment alters the alternative splicing pattern of *Fas* transcripts in HeLa cells

Of all the 19 proteins identified by 2DE coupled with MS analysis, up to 6 proteins are related to mRNA splicing (Table 1). It was therefore of great interest to determine whether splicing pattern changes

could be observed in HeLa cells in response to treatment with cisplatin. To address this question, we estimated the expression level of the two forms of *Fas* transcripts by RT-PCR analysis, as suggested previously by Filippov et al. [27]. The results (Fig. 6) demonstrate that expression of the soluble isoform of the *Fas* gene decreased in HeLa cells incubated with cisplatin for 5 h as compared to untreated HeLa cells, while expression of the membrane-bound isoform did not change appreciably in response to cisplatin treatment. These data, therefore, demonstrate that changes in the pattern of alternative splicing for *Fas* indeed occur in HeLa cells in response to cisplatin treatment.

#### 4. Discussion

Cisplatin is one of the most effective anticancer agents used in clinical chemotherapy of solid tumors, and its cytotoxicity is generally attributed to the generation of DNA damage caused by intrastrand or interstrand crosslinks [2–4]. However, the mechanisms underlying cisplatin-induced DNA damage and the corresponding cellular response are not yet fully understood. For this reason, we performed 2DE coupled with mass spectroscopic analysis on the HeLa nuclear proteome from treated and untreated cells in order to identify proteins involved in the action of cisplatin on the nucleus, and to suggest novel mechanisms of action. The high-throughput and systematic characteristics of proteomic technology has made it a very powerful, yet convenient method for many biological studies, which, in our case, is the study of mechanisms underlying DNA damage induced by cisplatin and the corresponding cellular response. Following 2DE separation and analysis by MS, we identified 19 out of 35 proteins that are differen-



**Fig. 5.** Immunofluorescent images of PSP1 in HeLa cells treated or untreated, with cisplatin. After 12 h treatment of 0.1, 1, 5 or 10  $\mu$ M cisplatin, cells were fixed and stained. Cells treated with 0.9% sodium chloride were used as control. The  $\gamma$ H2AX foci and PSP1 proteins were labeled with FITC and Cy3, respectively, with the nuclei stained with DAPI. The fluorescent images of Cy3 merged with those of FITC indicated that PSP1 does not colocalize extensively with  $\gamma$ H2AX foci.

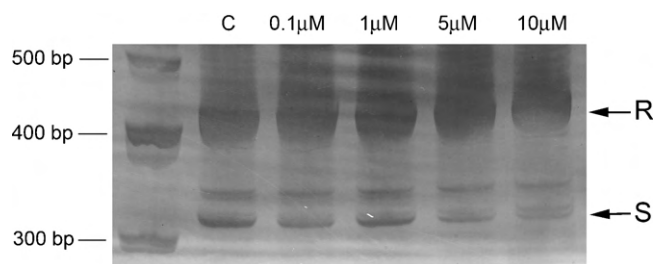
tially expressed in the HeLa nuclear proteome following treatment with different concentrations of cisplatin for 12 h. These proteins function in various biological processes, including biosynthesis, mRNA processing and signal transduction.

Of these 19 proteins, we found that six were related to the regulation of mRNA splicing. These include two members of the hnRNP family, paraspeckle component 1 (PSP1), splicing factor 45, far upstream element-binding protein 2 (KHSRP) and ATP-dependent RNA helicase DDX39 (DDX39) (Table 1). As opposed to the well-established mechanisms of transcriptional regulation, the mechanisms of alternative splicing are poorly understood. Alternative splicing plays an important role in all or nearly all biological processes in eukaryotes, and is under the precise control of numerous splicing factors [27]. Moreover, it has been suggested by recent studies that alternative splicing can be induced by DNA damage, and that this induction is under the regulation of splicing factors [32,33]. Alternative splicing is regarded as a widespread

phenomenon, in part due to the estimate that 74% of multi-exon genes are alternatively spliced [34]. Interestingly, these isoforms often function oppositely [34], and this may help to explain the complicated cellular response to DNA damage.

Splicing factors can be classified as belonging to one of two general classes: SR proteins and heterogenous nuclear ribonucleoproteins (hnRNP). Interestingly, the hnRNP H and hnRNP C proteins identified in this study belong to the hnRNP family. A number of studies have examined the alteration of hnRNP expression in response to cisplatin treatment, including hnRNP G, hnRNP H, hnRNP I and hnRNP K [19,20]. The hnRNP family is a collection of proteins that can bind to unspliced pre-mRNA. However, the members not only exhibit little similarity in protein structure and function, but also vary widely in their ability to carry out activities involved in RNA binding and oligomerization [35]. Here we found that the expression of hnRNP H and hnRNP C were up-regulated and down-regulated, respectively, by cisplatin treatment in HeLa cells, which was consistent with these general observations.

According to Filippov et al., *Fas* and *CD44* can be alternatively spliced under mechanisms triggered by mitomycin C treatment, which is, just like cisplatin, an anticancer drug and also a cross-linking agent [27,32]. Thus in order to determine whether the differential expression of splicing factors changed the splicing pattern, RT-PCR analysis was performed on *Fas* and *CD44* transcripts. Previously, change in splicing pattern of *CD44* but not that of *Fas* was observed in HeLa cells after benzo[a]pyrene treatment [36]. Here we found decreased soluble isoform of *Fas* transcripts in cisplatin-treated HeLa cells, while the splicing pattern of *CD44* remained unchanged (data not shown). Although there is difference between these results, which might contribute to the different stimuli used, both our studies suggest the occurrence of alternative splicing as a result of the genotoxin exposure. Other than the RT-PCR analysis of



**Fig. 6.** *Fas* splicing patterns change during the early response of HeLa cells to cisplatin treatment. Cells were treated with 0.1, 1, 5 or 10  $\mu$ M cisplatin for 5 h, and *Fas* expression was analyzed by RT-PCR analysis. The arrow R and S indicates membrane-bound receptor and soluble isoform, respectively.

*Fas* and *CD44*, a high-throughput detection of alternative splicing was performed using the AffyMetrix human Exon 1.0 ST array in our laboratory, and preliminary results revealed hundreds of genes alternatively spliced as a result of genotoxin treatment (data not shown). All these results suggested that cisplatin treatment did alter the splicing pattern, likely due to differential expression of one or more splicing factors.

There have been several reports that members of the intermediate filament family can be affected by cisplatin treatment [18–20,37,38]. In this study, we identified two lamins, Lamin A/C and Lamin B, which are components of a fibrous network named nuclear lamina and both could be repressed by cisplatin [28]. Previous studies showed that mutations of Lamin A might impair the ability of cells to form DNA repair foci containing  $\gamma$ H2AX in response to DNA damage, suggesting an important role for Lamin A in the DNA damage response [28,29]. Recent studies also found that loss of Lamin A hindered the processing of dysfunctional telomeres, which is putatively through stabilization of 53BP1, an important factor in DNA damage response pathway, thus suggesting a key role for Lamin A in the maintenance of genome integrity and the DNA damage response [39].

The mitotic checkpoint protein BUB3, when combined with a set of highly conserved proteins including BUB1, can function as a member of an important signal transduction pathway that mediates the spindle assembly checkpoint [40–42]. The cross-talk between the spindle assembly checkpoint and the DNA damage checkpoint pathways has been well-established, and PIKKs (PI3K-related kinase) are required to activate both [30]. Thus, BUB3 might participate in the DNA damage response as a consequence of its role in regulating the cell cycle.

Of significant interest was our identification of PSP1, a component of a nuclear body called the paraspeckle, which is adjacent to SC35 splicing speckles [21]. PSP1 contains two copies of the RNA recognition motif (RRM), which is the most prevalent RNA-binding domain in eukaryotes and a prerequisite for the localization of PSP1 to paraspeckles. Another two proteins, PSP2 and p54nrb, also contain two RRMs and together with PSP1, comprise the paraspeckles in HeLa cells [21]. Although there is no evidence of its direct involvement, p54nrb has been implicated in mRNA splicing because of its interaction with protein-associated splicing factor (PSF) which is well-known for its participation in mRNA splicing *in vitro* [43]. Thus, it has been suggested by Fox et al. that PSP1 might also be involved in the regulation of mRNA splicing [21]. The RNA-binding activity of PSP1 was confirmed by Myojin et al. in mouse Sertoli cells, and further study suggested that PSP1 might regulate androgen receptor-mediated transcriptional activity [44,45]. All the above studies suggest that PSP1 might be involved in RNA metabolism and processing.

Although there is little evidence that PSP1 is involved in the DNA damage response, it has been reported that PSP1 can be phosphorylated by ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad3-related) at Ser-509 [31]. Since ATM and ATR, members of the PI3K family, are well known as checkpoint proteins that can phosphorylate and ultimately catalyze the activation of the effector kinases in response to DSBs [46], the presumed phosphorylation of PSP1 by ATM/ATR indicates its possible involvement in the DSB response.  $\gamma$ H2AX recruits repair proteins and signaling factors involved in the response to DSBs, and these proteins together with  $\gamma$ H2AX form foci that are easily detected in cells [12–14]. Following simultaneous immunofluorescence detection of  $\gamma$ H2AX and PSP1, we found no obvious colocalization of PSP1 and  $\gamma$ H2AX foci in HeLa cells, either treated or untreated with cisplatin for 12 h (Fig. 5). Thus, PSP1 might not be recruited by  $\gamma$ H2AX or be involved in the early response to DSBs. However, we cannot exclude the possibility that PSP1 might function in some unknown response pathways. In addition, we have not yet examined the expression and distribu-

tion of phosphorylated PSP1, an approach that has the potential to provide us with additional evidence regarding its possible role in the DSB response.

The proteins identified in our study as part of the nuclear response to cisplatin treatment also include proteins involved in biosynthesis and proteins involved in posttranslational modification. Many or all are known to play important roles in fundamental biological activities, and several are differentially expressed in response to cisplatin treatment. Considering the complexity of the mechanisms underlying the DNA damage response, the effector proteins may indeed be involved in multiple biological activities. Since products of alternative splicing often possess different, even opposite functions [47–49], the change of splicing patterns we observed in cisplatin-treated HeLa cells is likely to play a significant role in the complicated DNA damage response. Together, these studies provide evidence that proteins involved in mRNA processing and splicing, the cytoskeleton, cell cycle, are either up- or down-regulated by DNA damage induced by cisplatin, suggesting that these and other pathways may be involved in the DNA damage response. However, there are still several questions regarding this study. For example, nearly half of the differentially expressed proteins were not successfully identified, and these unidentified proteins possibly have more important functions. Also, it should be kept in mind that this work is a tentative exploration of the mechanisms underlying the cytotoxic effects of cisplatin, further study is needed to clarify the specific role of those identified proteins and the corresponding pathways play in the DNA damage response.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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