REPORT

Translational regulation of the mRNA encoding the ubiquitin peptidase USP1 involved in the DNA damage response as a determinant of Cisplatin resistance

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ABSTRACT

Cisplatin (cis-diaminedichloroplatin (II), CDDP) is part of the standard therapy for a number of solid tumors including Non-Small-Cell Lung Cancer (NSCLC). The initial response observed is in most cases only transient and tumors quickly become refractory to the drug. Tumor cell resistance to CDDP relies on multiple mechanisms, some of which still remain unknown. In search for such mechanisms, we examined the impact of CDDP on mRNA translation in a sensitive and in a matched resistant NSCLC cell line. We identified a set of genes whose mRNAs are differentially translated in CDDP resistant vs. sensitive cells. The translation of the mRNA encoding the Ubiquitin-Specific Peptidase 1 (USP1), a Ubiquitin peptidase with important function in multiple DNA repair pathways, is inhibited by CDDP exposure in the sensitive cells, but not in the resistant cells. This lack of down-regulation of USP1 expression at the translational level plays a primary role in CDDP resistance since inhibition of USP1 expression or activity by siRNA or the small molecule inhibitor ML323, respectively is sufficient to re-sensitize resistant cells to CDDP. We involved the USP1 mRNA translation as a major mechanism of CDDP resistance in NSCLC cells and suggest that USP1 could be evaluated as a candidate predictive marker and as a therapeutic target to overcome CDDP resistance. More generally, our results indicate that analysis of gene expression at the level of mRNA translation is a useful approach to identify new determinants of CDDP resistance.

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Introduction

CDDP is part of the standard first line therapy to several solid cancers including lung, ovarian, colorectal, head and neck, bladder and testicular cancer. With the exception of testicular germ cell cancer to which CDDP is used with a curative aim, the initial tumor response to CDDP is nearly invariably followed by the emergence of resistance to the cytotoxic effect of the drug. For the treatment of solid tumors such as the NSCLC, CDDP resistance and the ensuing disease relapse is of particular clinical relevance and the understanding of the resistance mechanisms has been a major quest ever since CDDP was introduced into the clinic.

Thus, the most advanced high throughput technologies have enabled the exploration of the cellular traits associated with increased resistance to CDDP at multiple levels. For instance, the genomic and epigenetic features associated with the loss of CDDP sensitivity have been thoroughly examined through the search for Small Nucleotide Polymorphism (SNP) associated with CDDP resistance^{1–4} or by exome sequencing.⁵ Gene expression programs associated with CDDP resistance have also been intensively scrutinized by cDNA microarrays analysis or exploring alternative levels of gene expression regulation such as those mediated by microRNAs or long non-coding RNAs.^{6–9} Besides, functional screenings have been performed using small interfering RNA (siRNA)-mediated gene silencing

or chemical library screens in order to identify cellular functions conferring resistance to CDDP.^{10,11} Finally, some mechanism-driven, candidate-gene strategies have enabled the identification of ERCC1, whose gene product is involved in DNA repair, as a potential marker of non-small-cell lung cancer response to CDDP.¹² Overall, mechanisms of CDDP resistance are now better understood. Some gene expression or SNP signatures have been identified as potential predictors of CDDP sensitivity and functional screenings have led to the identification of promising candidate targets even though those findings still await clinical validation.

In order to identify new molecular events associated with CDDP resistance, we analyzed the regulation of gene expression at the mRNA level upon exposure of cells to CDDP for the following reasons. First, DNA damage such as those triggered by UV treatment lead to specific regulation of mRNA translation.^{13–16} Second, the regulation of gene expression is perhaps best reflected by its peptide product(s) level. The proteome, however, is still far less accessible compared to the transcriptome, the genome or the epigenome. As a result, only a few studies have examined the proteome associated with resistance of NSCLC to CDDP.^{17,18} Experimental strategies have however been devised to circumvent the technical hurdle of global protein level analysis. For instance, the ‘nascent translome’ is experimentally accessible by resolving cellular mRNA

according to their translation rate. Indeed an untranslated mRNA present as a ribonucleotide particle has a different sedimentation coefficient from polysome, i.e an mRNA associated with ribosomes.¹³ Therefore, untranslated mRNA can be separated from the actively translated mRNA by virtue of their sedimentation properties. cDNA microarray analysis of highly

translated vs untranslated mRNA can then be performed in different experimental conditions and enables the mRNA translation rates to be assessed.¹⁹ We implemented this approach in order to reveal the changes in protein synthesis associated with the response to CDDP of a sensitive vs. resistant NSCLC cell lines.²⁰

Giving an initial focus to the DNA repair processes, we identified a number of mRNAs regulated specifically at the translation level in the CDDP-resistant NSCLC cell line, among which the mRNA encoding the Ubiquitin-Specific Peptidase 1 (USP1). In complex with UAF1 (USP1 associated factor 1), USP1 catalyzes the removal of a monoubiquitin from its target proteins, 2 of which, FANCD2 and PCNA, are involved in DNA repair-associated processes.^{21,22} While the monoubiquitination of FANCD2 is required for its recruitment to chromatin, the USP1/UAF1-mediated removal of the FANCD2 monoubiquitin was shown to be required for the repair process to proceed. As a result, disruption of USP1/UAF1 complex's function leads to sensitization to crosslinking agents.^{23,24} In the case of PCNA, its deubiquitination by USP1/UAF1 is proposed to act as a switch between the translesion synthesis (TLS) DNA polymerase and the regular, more accurate DNA polymerase.²⁵⁻²⁷

In this study, we identified an altered regulation of USP1 mRNA translation in CDDP-resistant cells and explored its contribution to CDDP resistance in NSCLC cells.

Results

Identification of translationally regulated mRNA involved in resistance to CDDP

In search for novel genes involved in CDDP resistance, we used a set of 2 matched human NSCLC cell lines: the relatively CDDP-sensitive A549 and an A549-derived cell clone, clone A549-R that has acquired CDDP resistance through long-term exposure to the drug.²⁰ Indeed the A549-R cells show a 3-time increase of their IC50 (90 μ M) compared to the parental A549 cells (30 μ M) (Fig. 1A).²⁰

To identify mRNAs regulated at the translational level by CDDP treatment, we performed a cDNA microarray analysis of mRNAs present in polysomes. In brief, the cells were exposed to 30 μ M CDDP for 4 and 16 hours. Cytoplasmic cell extracts were then prepared and fractionated into 22 fractions on a sucrose gradient. The high density fractions (fractions 19 to 22) containing the highly translated mRNA (polysomal

Figure 1. The CDDP-induced translational regulation of USP1 mRNA is lost in the resistant cell line (A) A549 and A549-R cells survival was measure after 48 hours exposure to CDDP using a colorimetric proliferation assay. The IC50 of CDDP determined by non-linear fitting of the absorbance is 30 μ M for the A549 cell line and 90 μ M for the A549-R cells. Error bars represent standard error of the mean. (B) Cell extracts from CDDP- or vehicle-treated A549 and A549-R cells were separated on a sucrose density gradient and collected into 22 fractions across the gradient. mRNA was isolated from individual fractions 8 to 22 and qPCR was performed using primers specific to USP1 mRNA. The percentage of USP1 mRNA in each fraction was plotted. Error bars represent standard error of the mean. (C) Western blot detection of USP1 protein expression in A549 and A549-R cells treated with CDDP as indicated. GAPDH detection is used as a loading control. Band quantification and USP1/GAPDH ratio are shown underneath blots. (D) Western blot detection of USP1 and TP53 proteins in A549 and A549-R cells treated with cycloheximide (CHX) for the time indicated. β -Actin and GAPDH were used as loading controls. Bands were quantified and the percentage of signal relative to untreated conditions are shown underneath blots.

fraction) were pooled. Extracted RNAs from the polysomal fraction as well as total RNAs (Input) from untreated and CDDP-treated cells were hybridized on GE 8X60K microarray (Agilent). We selected genes for which mRNAs were differentially present in the polysomal fraction (PF) after 4 hours or 16 hours exposure to CDDP compared to control untreated cell extracts (limma test p -value < 0.05). mRNAs regulated in a similar fashion in the input fraction were filtered out in order to exclude transcriptional regulation. **Table 1** shows a list of DNA repair-associated genes that fit these criteria (see also Fig. S1). Of note, several FA genes or regulators of the FA pathway are regulated at the translational level upon CDDP exposure, some of them in a cell line specific manner (FANCB, FANCF and USP1). As opposed to FANCB and USP1, FANCF translation is slightly repressed in the resistant A549-R cells (**Table 1**).

CDDP-dependent translational regulation of USP1 mRNA in CDDP-sensitive vs. resistant cells

Among the genes identified in our microarray analysis, USP1 was of particular interest as several small molecule inhibitors of its activity have previously been developed.^{28,29} As our results suggest a differential regulation of the USP1 mRNA translational rate in the 2 cell lines, we further examined the levels of USP1 mRNA in each fraction individually (**Fig. 1B**). Strikingly, USP1 mRNA relocated from moderately translated (fractions 14 to 17) to untranslated fractions (fractions 12 to 14) after 4 hours exposure to CDDP in A549 cells. After 16 hours of CDDP exposure, the USP1 mRNA was predominantly found in the highly translated mRNA fractions (fractions 18 to 20), suggesting a biphasic regulation of USP1 mRNA translation. In the resistant A549-R cells, however, the regulation of USP1 translation is lost and the mRNA is distributed among the fractions in the same manner in CDDP-treated or untreated A549-R cells. The distribution of ABCA1 mRNA, taken as a control, was comparable in both cell lines, suggesting a specific alteration of USP1 mRNA translational regulation in the A549-R cells (**Fig. S2**).

To investigate whether the effect of CDDP treatment on USP1 mRNA translation rate impacted on the gene's protein

product levels, we performed Western blot analysis of USP1 protein in A549 and A549-R cells treated with CDDP for various times. After a slight decrease, USP1 protein levels increased at a later time point upon CDDP exposure in the A549 cells, but not in the A549-R cells, confirming the mRNA translation analysis (**Fig. 1C**).

Finally, as cellular protein level is the result of both synthesis and degradation, we ascertained that USP1 protein stability was not altered in the A549-R cells. For that purpose, we treated both cell lines with the protein synthesis inhibitor Cycloheximide (CHX) for various time in order to reveal differences in protein half-life in each cell line. TP53, a protein of notoriously short half-life, was monitored to ensure efficient protein synthesis inhibition in our experimental conditions (**Fig. 1D**). As shown in **Figure 1D**, USP1 protein displays a comparable half-life in the A549 and the A549-R cell lines.

Interfering with USP1 function sensitizes cells to CDDP

We next sought to evaluate the contribution of the regulation of USP1 expression to CDDP resistance. For that purpose, we used an siRNA targeting USP1 that lead to a modest but consistent downregulation of USP1 gene expression in both A549 and A549-R cells (**Fig. 2A**). We then assessed the sensitivity of these USP1-depleted cells to CDDP. While USP1 knock-down had no effect on A549 sensitivity to CDDP, the A549-R cells were partially but clearly re-sensitized to the drug after transfection with the USP1-targeting siRNA (IC₅₀ 90 μ M), but not with the control, non-targeting siRNA (IC₅₀ 50 μ M, **Fig. 2B**).

The siRNA-mediated knock-down of USP1 is only partial. We therefore tested the effect of the recently developed small molecule inhibitor of USP1 activity, ML323, on the sensitivity of A549 and A549-R cells to CDDP.²⁹ We first assessed the potency of ML323 in our cell models by examining the monoubiquitination of 2 USP1/UAF1 target proteins, PCNA and FANCD2. As previously reported, CDDP treatment induced a slight increase of the monoubiquitinated forms of the 2 target proteins (**Fig. 3A**).^{22,25-27} The ML323 treatment lead to an accumulation of the monoubiquitinated forms of PCNA and FANCD2 in basal and CDDP-treated conditions, confirming

Table 1. Identification of DNA repair genes differentially translated after cisplatin exposure by cDNA microarray. DNA repair related gene transcripts that were differentially detected in the polysomal fraction upon CDDP treatment (limma test p -value < 0.05) and not in the input (limma test p -value > 0.05) are shown. Genes in bold are differentially regulated in a cell-line specific manner and may therefore be associated to CDDP-resistance. HRR, Homologous Recombination repair, NHEJ, Non-Homologous End-Joining, NER, Nucleotide Excision Repair, BER, Base Excision Repair.

geneSymbol	Function	4H CDDP vs Untreated			16H CDDP vs Untreated		
		A549-R		A549	A549-R		A549
		FC.C4	pval.C4	FC.A549.FP.C4	FC.C16	pval.C16	FC.A549.FP.C16
FANCB	FANCD2 monoubiquitination	1.35	0.032	1.21	1.54	0.003	1.09
FANCF	FANCD2 monoubiquitination	1.01	0.873	-1.11	-1.26	0.047	1.06
USP1	FANCD2 monoubiquitination	-1.03	-0.736	-1.02	-1.35	-0.004	-1.03
BRIP1 (FANCF)	FA, HRR	-1.41	-0.029	-1.06	-1.01	-0.950	-2.12
POLD2	Replicative DNA pol	-1.04	-0.620	-1.10	-1.29	-0.031	-1.05
POLD4	Replicative DNA pol	-1.26	-0.022	-1.12	-1.34	-0.005	-1.62
POLL	NHEJ	-1.28	-0.007	-1.02	-1.04	-0.626	-1.29
FEN1	Flap endonuclease	-1.28	-0.158	-1.19	-1.41	-0.043	-1.59
CCNH	NER	-1.08	-0.548	-1.00	-1.42	-0.017	-2.01
POLB	BER	-1.12	-0.653	-1.11	-1.38	-0.023	-1.66
ZBTB32	Transcription factor	-1.12	-0.026	-1.16	-1.02	-0.703	-1.02

Figure 2. siRNA-mediated silencing of USP1 partially re-sensitize A549-R cells to CDDP. (A) RNAs from A549 and A549-R transfected with USP1-targeting or control siRNA were isolated. qPCR was performed to assess USP1 mRNA levels. Error bars represent standard error of the mean. (B) CDDP dose-response of A549 and A549-R cells transfected with control or USP1-targeting siRNA as indicated. Drug treatment was started 24 h after transfection. Cell survival was measured after 48 h exposure to CDDP using a colorimetric proliferation assay. The IC₅₀ of CDDP was determined by non-linear fitting of the absorbance. Error bars represent standard error of the mean.

that ML323 efficiently inhibits the USP1/UAF1 complex activity in our cell models (Fig. 3A).

We then evaluated the impact of ML323-mediated inhibition of USP1 function on the survival to CDDP treatment. A549 and A549-R cells were exposed to CDDP in the absence or in the presence of 30 μ M or 100 μ M ML323. As shown in Figure 3B, the presence of ML323 resulted in a dose-dependent sensitization of the A549 cells to CDDP. Most importantly, while the low dose of ML323 has no effect on A549-R cells resistance to CDDP, higher concentrations of the inhibitor re-sensitized the resistant cells to levels comparable to the parental A549 cell line (IC₅₀ 34 μ M with ML323 at 100 μ M, Fig. 3B). Taken together, these results suggest that USP1 function is a major determinant of CDDP cytotoxic activity in our cell models.

Resistance to CDDP is associated with shortening of USP1 mRNA 5' UTR

The resistance of the A549-R cells appears to be mediated in part by the deregulation of USP1 mRNA translation. The translation rate of an mRNA is mainly regulated at the initiation stage, which can be controlled, among other features, by the presence of *cis*-regulatory element in the 5'-untranslated region (5' UTR) of the gene transcript(s). USP1 gene can generate transcripts with differing 5' UTRs resulting from alternative promoter usage (see for example <https://fasterrdb.lyon.univ-cancer.fr>) (Fig. 4A). We performed an RT-PCR analysis of the 5' UTRs of the USP1 mRNAs in A549 and A549-R cells. We could detect 2 mRNAs potentially arising from alternative promoter usage within the exon 2 (PCR 1 and 2, Fig. 4A and

Fig. S3). Interestingly, the relative abundance of these mRNA variants was found to be cell line specific (Fig. 4B). While the transcription of the USP1 gene seems to arise predominantly from the second transcription start site downstream of the second promoter (P2) in A549 cells, the A549-R cells also express a shorter mRNA transcribed from the P3 promoter. Interestingly, when analyzed by an RNA secondary structure prediction software (see for example <http://mfold.rna.albany.edu>), the longer USP1 mRNA 5'UTR is predicted to have a higher minimum free energy (ΔG -314.50) than the shorter 5' UTR (ΔG -163.80) (Fig. S3). The initiation of the translation of the shorter 5' UTR may therefore be less prone to secondary structure-mediated repression. Alternatively, the USP1 mRNA bearing a shorter 5' UTR expressed in the A549-R cells could potentially lack *cis*-regulatory regions responsible for the translational regulation observed in the CDDP-sensitive A549 cells. These two levels of translational regulation are proposed as candidate mechanisms for the USP1 translational deregulation associated with CDDP resistance in the A549-R cells.

Discussion

Resistance to chemotherapeutic agents can involve various intrinsic cellular processes including drug efflux, increased resistance to apoptosis, or increased DNA repair capabilities in the case of platinum salts or other DNA damaging drugs. We chose to give an initial focus on the involvement of the DNA repair processes in resistance to CDDP. In this study we employed a high throughput approach designed to identify genes regulated at the translational level upon CDDP treatment. Performing this analysis concomitantly in a sensitive and a matched resistant cell line enabled us to identify mRNAs differentially regulated at the translation level by CDDP in sensitive vs. resistant cell lines. We specifically identified translationally-regulated mRNAs encoding DNA repair genes, among which USP1 was chosen for further investigation because previously developed small molecule inhibitors are available for pre-clinical studies as well as for potential clinical developments.^{28,29}

USP1 was shown to be involved in DNA repair as a deubiquitinating enzyme (DUB) for monoubiquitinated FANCD2.²² FANCD2 monoubiquitination is crucial to initiate DNA lesion processing and removal and is catalyzed by the FA core complex upon DNA damage in an ATR-dependent process.³⁰ Interestingly, 2 FA core complex genes required for FANCD2 monoubiquitination, FANCB and FANCF, were also identified in our screen (Table 1 and Fig. S1).^{31,32} In a subsequent step of the FA pathway activation, the USP1/UAF1-mediated removal of the ubiquitin moiety is required for FANCD2 to delocalize away from the chromatin and for the FA pathway to proceed.^{23,24} Thus, USP1/UAF1 activity does not antagonize the FA core complex ubiquitin-ligase activity, but instead enable the FA pathway to proceed and triggers DNA lesion processing. In the sensitive A549 cell line, USP1 protein levels initially drop slightly upon CDDP exposure, potentially enabling FANCD2 monoubiquitination. Later on, the USP1 protein level rises through increased mRNA translation rate. This identifies mRNA translation as a candidate mechanism for the transient and timely control of FANCD2 monoubiquitination (Fig. 1B-C).

Figure 4. Alternative USP1 mRNA 5'-UTR in the resistant A549-R cells (A) Schematic representation of the 5'-region of USP1 transcript. Exons are represented as boxes. Intronic sequences are represented by solid lines. Red dotted line represents alternative splicing. Putative promoters are denoted by black arrows and the translation start site by a black stars. Position of the primers used in the PCR analysis are shown (colored arrows). (B) Total RNAs from A549 and A549-R cells treated with CDDP 30 μ M or with vehicle for 16 h were analyzed by semi-quantitative RT-PCR using primers as depicted in Figure 4A. Band quantification is shown underneath blots.

Beside the increased levels of USP1 in the resistant A549-R cells, the levels of both unmodified and monoubiquitinated FANCD2 are also clearly much higher in these cells compared to the sensitive cell line. Taken together, these data point to an increase activity of the FA pathway in the resistant A549-R cells and suggest that ICL, one of the platinum-induced DNA lesions that are processed by FA, may be a crucial determinant of CDDP cytotoxic activity in this cell model.

Another crucial function of the USP1/UAF1 complex is the removal of PCNA monoubiquitination that regulates the recruitment of translesion synthesis (TLS) DNA polymerase.²⁵⁻²⁷ TLS through damaged DNA can participate in cell resistance to chemotherapeutic agents and the USP1 deregulation in our resistant cell model may well impact on TSL polymerase

activity, even though further investigation is needed to test this possibility.

In agreement with previous reports, we found that interfering with USP1 function using siRNA or a small molecule inhibitor was sufficient to re-sensitize the resistant cells to CDDP.^{28,29} This opens the possibility of targeting USP1 activity using small molecule inhibitors to circumvent resistance to CDDP, even though this remains to be clinically assessed.

In this study, we report that the resistant A549-R cells express a shortened USP1 mRNA. As USP1 translational regulation is lost in these cells, we hypothesized that this short mRNA may be less prone to secondary structure-mediated translational repression or may lack regulatory sequences in its 5'-UTR. The trans-acting factor(s) that potentially fail to bind

the regulatory region of USP1 mRNA remain(s) to be identified. Moreover, whether this level of regulation is commonly disrupted in CDDP-resistant NSCLC cell lines is an important question that still needs to be addressed. More generally, to our knowledge, USP1 gene expression has not been studied at the translational level and it remains to be determined whether the mechanism described in this study may be generalized across additional cancer cell lines. Nevertheless, as the total mRNA levels of USP1 are stable in our resistant and the sensitive cells lines, and as USP1 protein detection in lung tumor samples gives conflicting results, we propose the assessment of the USP1 5'-UTR as an alternative predictive marker of CDDP resistance easily amenable to routine practices.^{33,34}

Material and Methods

Cell culture, siRNA transfection, drug treatment and colorimetric proliferation assay

The A549 and A549-R NSCLC cell lines were maintained in DMEM supplemented with 10% Fetal Calf Serum, Penicillin (100 U/mL) and Streptomycin (100 µg/mL) at 37°C, 10% CO₂ in humidified atmosphere. The A549-R cell line was derived from the A549 cell line by chronic exposure to CDDP as previously described.²⁰

siRNA transfection was performed using Lipofectamine[®] RNAiMAX Reagent following manufacturer instructions. siRNA were from Santa Cruz biotechnology, INC (Control siRNA, sc-37007 and USP1 siRNA, sc-88494).

Proliferation was measured using the Cell Proliferation Reagent WST-1 (Roche) according to manufacturer's instructions.

The USP1 inhibitor ML323 was from Axon Medchem (2309). Cycloheximide was purchased from sigma and used at a final concentration of 300 µg/mL.

Polysome profiling and RT-PCR

A549 and A549-R cells were treated with vehicle or with CDDP at 30 µM for 4 h or 16 h. Cells were treated with cycloheximide at 100 µg/mL for 15 min to avoid ribosome off loading, then rinsed twice with cold PBS supplemented with 100 µg/mL cycloheximide and harvested by scrapping on ice. Every further steps were performed on ice whenever possible. Cell pellet was resuspended in 1 volume of LSB buffer (20 mM Tris pH7.5, 100 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 100 U/mL RNasin[®] Plus RNase inhibitor (Promega), 100 µg/mL cycloheximide). One volume of LSB-ST buffer (LSB supplemented with 0.5 M sucrose and 2.4% TritonX100) was then added, the cell suspension was kept on ice for 30 min before centrifugation at 12,000xg at 4°C for 10 min. Supernatant was collected and salt concentrations were adjusted to NaCl 150 mM and MgCl₂ 10mM. Samples were snap-frozen in liquid nitrogen if not processed immediately.

An aliquot of 1/10 of the cell extract was spared as the Input sample. The remaining extract was loaded on a sucrose density gradient ranging from 15% to 50% sucrose in LSB buffer. Sample separation was performed by ultracentrifugation at

38,000xg for 2 h at 4°C. Samples were fractionated across the density gradient using an Isco gradient fractionation system.

For the cDNA microarray analysis, mRNA from the 4 heaviest fractions were pooled (polysomal fraction, FP) and from the total cells extract (Input) were prepared using the TRizol[®] RNA Isolation Reagent (Life technologies) according to manufacturer's instructions. Quadruplicates were analyzed on a GE 8X60K (Agilent) microarray platform.

The same samples were also reversed transcribed using Mx Reverse Transcriptase (Life Technologies) and Random hexamers and analyzed by semi-quantitative RT-PCR (GoTaq[®] Hota Start Polymerase, Promega) for validation of the microarray data and the analysis of the USP1 mRNA 5'-UTR using primers listed in Table S1.

For polysome profiling, mRNA was isolated using TRizol[®] LS Reagent (Life technologies) according to manufacturer's instructions, reverse transcribed and analyzed by qPCR using Thermo Scientific[™] Luminaris[™] Color HiGreen HiRox master mix and the primers listed in Table S1.

Statistical tests

Differential expression was computed using the limma package in R that estimates the fold changes and standard errors by fitting a linear model for each gene and applying empirical Bayes smoothing to the standard errors.³⁵

Antibodies and Western blot analysis

Cells were washed in PBS, harvested by scrapping, pelleted and lysed in RIPA buffer supplemented with cOmplete (Roche) protease inhibitors cocktail for 30 min on ice. After a centrifugation at 20,000xg for 15 min at 4°C, the supernatant was used as a protein extract. Protein extracts were resolved by SDS-PAGE in 10% Bis-Tris acrylamide (for USP1 and PCNA) or 3%-8% Tris-Acetate acrylamide (FANCD2) gels, transferred onto a nitrocellulose membrane and probed with the following antibodies in TBS, 5% milk, 0.05% Tween[®] 20: anti-USP1 (Abcam ab108104, 1/1,000), anti-PCNA (Santa Cruz biotechnology, INC, cs-56, 1/1,000), anti-FANCD2 (Santa Cruz biotechnology, INC, sc-20022, 1/1,000), TP53 (Santa Cruz biotechnology, INC, sc-47698, 1/1,000), anti-β-Tubulin (Sigma, T8328, 1/1,000), anti-β-Actin (Sigma, A5441, 1/1,000) and anti-GAPDH (Millipore, MAB 374, 1/2,000). ECL was performed using SuperSignal[™] West Pico Chemiluminescence Substrate (Life technologies) according to manufacturer's instructions. Bands were quantified using the Image Lab 5.1-Beta software (Bio-Rad Laboratories).

Abbreviations

5' UTR	5'-Untranslated Region
ABCA1	ATP-binding cassette, sub-family A, member 1
CDDP	cis-diaminedichloroplatin (II)
ERCC1	Exision Repair Cross-Complementation group 1
FA	Fanconi Anemia
FANCB	Fanconi Anemia complementation group B
FANCD2	Fanconi Anemia complementation group D2
FANCF	Fanconi Anemia complementation group F

NSCLC	Non-Small-Cell Lung Cancer
PCNA	Proliferating Cell Nuclear Antigen
SNP	Small Nucleotide Polymorphism
TLS	Trans-Lesion Synthesis
UAF1	USP1 associated factor 1
USP1	Ubiquitin-Specific Peptidase 1.

Disclosure of Potential Conflicts of interest

No Potential Conflicts of interest were disclosed.

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