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UBE2M-mediated p27^{Kip1} degradation in gemcitabine cytotoxicity

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ABSTRACT

Gemcitabine (2′-deoxy-2′, 2′-difluorocytidine; Gem) is a nucleoside anti-metabolite and is commonly used for treating various human cancers including human bladder carcinoma. Gemcitabine not only functions as a suicide nucleoside analog but also inhibits DNA polymerase activity and results in the termination of chain elongation. Using 2-dimensional gel electrophoresis analysis, a Gem-induced protein was identified as UBE2M (a.k.a. UBC12), a NEDD8 conjugation E2 enzyme which contributes to protein degradation. Gem induced UBE2M expression at both RNA and protein levels in several human cancer cell lines. The induction of UBE2M by Gem was accompanied by a reduction in p27^{Kip1} protein levels, which could be restored by silencing UBE2M expression with siRNA or by treating cells with the proteasome inhibitor MG132, indicating that UBE2M mediates Gem-induced p27^{Kip1} protein degradation. The induction of UBE2M and reduction of p27^{Kip1} by Gem were prevented by the PI3K inhibitor LY294002. These results indicate that PI3K activity is necessary for Gem-induced UBE2M expression and that UBE2M facilitates degradation of p27^{Kip1}. Notably, silencing of UBE2M expression reduced Gem sensitivity in NTUB1 cells, suggesting that UBE2M mediates in part cell sensitivity to Gem, possibly by degradation of p27^{Kip1}. Analysis of Gem-resistant sub lines also showed that loss of UBE2M and increased p27^{Kip1} expression were associated with the acquisition of drug resistance. In conclusion, our results demonstrate a role for UBE2M in mediating cytotoxicity of gemcitabine in human urothelial carcinoma cells while also suggesting a potential function of p27^{Kip1} in drug resistance.

1. Introduction

Urinary bladder cancer is the fourth respectively eighth most common cancer in men and women in the United States [1]. Urothelial carcinoma (UC) is the most common type of bladder cancers, accounting for more than 90% of all bladder cancers [2,3]. According to the Cancer Registry issued by the Department of Health in Taiwan for 2009, UC ranks as in the 14th most common cause of cancer death and the death rate per 100,000 of the population was accounted for 1.9% in Taiwan. The treatment strategy for UC is mainly surgery followed by chemotherapy or radiotherapy [4]. Chemotherapeutic agents are active in advanced bladder tumors, and various combinations have shown promising results [5,6]. M-VAC regimen (methotrexate, vinblastine, adriamycin and cisplatin) has been used to treat UC patients since 1985 but is associated with significant morbidity [7]. A gemcitabine (2′-deoxy-2′, 2′-difluorocytidine; Gem) plus cisplatin (GC) regimen showed promising results in advanced and metastatic bladder tumors and has been widely used [7,8]. While progress has been made, more knowledge is necessary to make predictions for and understand the clinical response and outcome to chemotherapy [9]. Gem is a deoxycytidine analog and has to be activated through various cellular enzymes to be incorporated into DNA [10]. Gem not only functions as a suicide nucleoside analog but also inhibits DNA polymerase and results in the termination of chain elongation

Abbreviations: UBE2M, ubiquitin-conjugating enzyme E2M; Gemcitabine (Gem), 2′-deoxy-2′, 2′-difluorocytidine; NEDD8, neural precursor cell-expressed developmentally down-regulated 8; SRM, spermidine synthase; PMVK, phosphomevalonate kinase; DLST, dihydrolipoamide S-succinyltransferase; UROD, uroporphyrinogen decarboxylase; FASN, fatty acid synthase; PSMB6, proteasome (prosome, macropain) subunit, beta type, 6.

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[11]. Despite these promising results, however, the mode of action and the underlying molecular mechanisms by which gemcitabine acts as a chemotherapeutic agent still need further investigations.

Alterations in cell cycle regulation are common in cancer progression. $p27^{Kip1}$, a cyclin E/CDK2 kinase inhibitor, was initially identified as an inhibitor of the G1 to S phase progression in cells arrested by transforming growth factor-β [12]. $p27^{Kip1}$ has been suggested as a potential prognostic factor for response to chemotherapy [13]. $p27^{Kip1}$ protein turnover is mainly regulated by ubiquitin-mediated proteolysis through the SCF^{Skp2} complex [14] which depends on an ubiquitin-like protein NEDD8 (neural precursor cell-expressed developmentally down-regulated 8) conjugating pathway including UBE2M [15]. Covalent conjugation of NEDD8 to cullins plays an important role in activation of ubiquitin-mediated protein degradation pathways [16,17]. UBE2M (ubiquitin-conjugating enzyme E2M; UBC12) is a 138 amino acids protein and functions as a unique E2 ubiquitin-conjugating enzyme for NEDDylation [18]. Ubiquitin and ubiquitin-like proteins control many cellular functions including proliferation, apoptosis, DNA repair responses, and the cell cycle [19].

The mechanism of Gem action is thought to involve the induction of prolonged cell cycle arrest, leading to cell death [11]. In this study, UBE2M was identified as a Gem-induced gene, which augments $p27^{Kip1}$ polyubiquitination and downregulation, and contributes to Gem cytotoxicity, providing new insights into the mechanisms of gemcitabine action on tumor cells.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against specific proteins were as follows: UBE2M (SC-100608, Santa Cruz Biotechnology, Santa Cruz, CA, USA), $p27^{Kip1}$ (#610241, BD Transduction Laboratories, CA, USA), Cyclin D1 (SC-8396, Santa Cruz), CDK2 (#101226, Genetex), CDK4 (#102993, Genetex), AKT1/2/3 (SC-8312, Santa Cruz), p-AKT (Ser473)(#4060S, Cell Signaling Technology, Beverly, MA, USA), PI3K (06-195, Upstate Biotechnology, Lake Placid, NY, USA), p-PTEN (Ser380) (#9551, Cell Signaling), PTEN (GTX101025, Genetex), HA (SC-7392, Santa Cruz), β-actin (C-11) (#600-501, Novus Biologicals, Littleton, CO, USA), α-tubulin (#11304, Genetex), goat anti-mouse IgG-HRP (JAS111-035-003, Jackson Laboratory, Bar Harbor, ME, USA), and goat anti-rabbit IgG-HRP (JAS111-035-003, Jackson Laboratory). LY294002 and MG132 were purchased from Calbiochem (San Diego, CA, USA) and dissolved in DMSO (Sigma Chemical Co., St Louis, MO, USA.). Gemcitabine was obtained from Eli Lilly Company (Lilly France, Fegersheim, France). Enhanced chemiluminescence detection kit (ECL) was purchased from Amersham Life Sciences Inc. (Amersham, England).

2.2. Cell culture, transfection and establishment of gemcitabine-resistant sublines

NTUB1, a human urothelial carcinoma cell line, was established from a high-grade bladder cancer [20] and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin-G, 100 μg/ml streptomycin and 2 mM L-glutamine (all from Invitrogen). PC3 (a human prostate cancer cell line), and T24 (an urothelial carcinoma cell line) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as described for NTUB1 cells. PANC-1 (a pancreatic cancer cell line) was obtained from ATCC and maintained in DMEM medium (Invitrogen) supplemented as described above. All cells were grown at 37°C, 5% CO_2.

For transient transfection experiments, NTUB1 cells were transfected with vector or HA-ubiquitin expression construct by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

To generate gemcitabine resistant sublines, NTUB1 cells were chronically exposed to progressively increasing concentrations of gemcitabine. Three sublines that could survive at 0.6, 0.8 and 1.0 μM gemcitabine were established and designated as NTUB1/Gem0.6 (NG0.6), NTUB1/Gem0.8 (NG0.8) and NTUB1/Gem1.0 (NG1.0), respectively. The cells were maintained with indicated gemcitabine concentrations at 37°C, 5% CO_2. PANC-1 gemcitabine-resistant sublines, PANC-G100 (PG100), PANC-G300 (PG300) and PANC-G1500 (PG1500) were established similarly and maintained in DMEM medium with supplements and 0.33, 1.0 and 5.0 μM Gem, respectively (Liou, JY et al., to be described elsewhere).

2.3. Cytotoxicity analysis by MTT assay

Cellular cytotoxicity to gemcitabine was assessed by a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St Louis, MO, USA.) assay [21]. Briefly, the cells were plated at a density of 4×10^3 cells/well in 96-well plates and incubated at 37 °C overnight. Cells were then cultured in the presence of graded concentrations of gemcitabine at 37 °C for 72 h. Then, 50 microlitres of MTT (2 mg/ml in PBS) were added to each well and incubated for another 4 h. Following centrifugation of plates at $1000 \times g$ for 10 min, media were removed and 150 microlitres of DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540 nm using a microplate reader, MRX (DYNEX Technologies, Chantilly, VA, USA). The cell viability was expressed relative to the un-treated control. The IC_{50} values of each group were calculated by median-effect analysis and presented as mean ± standard deviation (SD).

2.4. Two-dimensional gel electrophoresis and image processing

The soluble proteins were isolated with lysis buffer (30 mM Tris-base, pH 8.0, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 100 μl/ml protease inhibitor cocktail (Sigma) and 0.5 mM PMSF). The proteins were processed and subjected to two-dimensional gel electrophoresis experiments with 13 cm Immobiline DryStrips (pH 3–10 NL) according to the manufacture's recommendations (GE Healthcare, Uppsala, Sweden). Isoelectric focusing was carried out using an IPGphor system (GE Healthcare) at 300 V for 3 h, 600 V for 1 h, 1100 V for 1 h, and a constant 8000 V until approximately 16000 V h was reached. The strips were equilibrated and were applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After SDS-PAGE, the gels were visualized by silver staining. The patterns of spots were analyzed with the Image Master 2D Platinum software version 5.0 according to the instructions. The relative intensity (%intensity) and relative volume (%vol) of each spot were calculated and normalized by the software to adjust for the variations in experimental conditions.

2.5. In gel digestion and protein identification by nanoESI-Q-TOF

Silver stained protein spots were excised, destained, washed and dehydrated in acetonitrile for 20 min. The solution was then aspirated and gel pieces were then brought to complete dryness. Gel pieces were rehydrated with a freshly prepared solution of sequence-grade modified trypsin (20 ng/μl in a 25 mM ammonium bicarbonate buffer) at 37 °C for 16 h. The tryptic peptides were extracted with a 50% acetonitrile/5% formic acid mixed solution by sonication. The extracted solutions were evaporated and then

re-dissolved in 10 μl 0.1% trifluoroacetic acid. The samples were then subjected to Waters-Micromass electrospray ionization quadrupole-time of flight (ESI-Q-TOF) for protein identification (Waters, Manchester, UK) as described previously [22]. Individual fragment spectra obtained for each of the precursors were processed by using MassLynx 4.0 software (Waters) to obtain centroid MS/MS data and then submitted to the MASCOT (http://www.matrixscience.com) search engine to find the corresponding protein identity.

2.6. Western blot analysis and immunoprecipitation

For Western analysis, cells were harvested by trypsinization and resuspended in a suitable amount of PBS adjusted by the cell numbers as described previously [21]. The cells were mixed with an equal volume of 2× sample buffer and boiled for 5 min twice to denature the proteins. The cell extracts were then separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA) using a semi-dry blotter (GE Healthcare). The blotted membranes were blocked with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20) and the membranes were incubated with specific antibodies at 4 °C overnight. The membranes were washed with TBST buffer and incubated with the secondary antibody at room temperature for 1 h. Proteins were then detected by chemiluminescence ECL reagent after TBST washing and visualized on Fuji SuperRX film.

For immunoprecipitation, proteins were extracted by RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitor cocktail (Calbiochem)). One microgram HA antibody was mixed with 1 mg cellular proteins and rotated overnight at 4 °C. Fifty microlitres Protein G (Santa Cruz) were added to the mixture for 2 h at 4 °C before centrifugation at 10,000 rpm for 1 min, followed by three washes with lysis buffer. Fifty microlitres 2× sample buffer were then added to each IP mixture and denatured in 100 °C for 5 min for SDS-PAGE and Western analysis as indicated.

2.7. UBE2M siRNA and transfection

Two siRNA oligos directed against the human *UBE2M* (accession number NM_003969.3) ORF as indicated below were purchased from Invitrogen.

siUBE#1: 5′-GGGCTTCTACAAGAGTGGGAAGTTT-3′ (nt 799-823),
siUBE#2: 5′-TCTACAAGAGTGGGAAGTTTGTGTT-3′ (nt 804-828).

Non-specific scramble siRNA was obtained from Invitrogen and used as control. 50 nM siRNA were transfected into NTUB1 cell using the lipofectamine RNAiMAX (Invitrogen) following the manufacture's instructions. Twenty-four hours post-transfection, the cells were treated as indicated.

2.8. RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's recommendations. Two micrograms of total RNA were used for reverse transcription using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems; ABI, Foster City, CA, USA) following the manufacturer's instructions. For real-time qPCR, the ABI PRISM 7900 Sequence Detection System (ABI) was used. Nine microlitres of master-mix (1X LightCycler Fast Start DNA Master SYBR Green I (ABI), 4 mM $MgCl_2$, 0.4 micromolar forward primer and 0.4 micromolar reverse primer) and 1 microlitre of 50 nanograms cDNA were added to the 96-well plates and amplified using a suitable program. At the completion of cycling, melting curve analysis was performed to establish the specificity of the amplicon production. The levels of expression of each mRNA and their estimated crossing points in each sample were determined relative to the standard preparation using the Light Cycler computer software. Cyclophilin A gene amplification was served as an internal normalization control. The expression levels of each gene relative to control were estimated by calculating $\Delta\Delta C_t$ ($\Delta C_{t,UBE2M} - \Delta C_{t,cyclophilin\ A}$) and subsequently using the $2^{-\Delta\Delta C_t}$ method [23]. The relative abundances of the mRNA were expressed as fold change compared to controls. All samples were independently analyzed at least twice for each gene.

The primers used were:

UBE2M-UTR for: 5′- AGAAGAAGGAGGAGGAGTC -3′
UBE2M-UTR rev: 5′- AGTTGAGGAGGTCGTCTG -3′
p27^{Kip1} for: 5′-GCGCAGGAATAAGGAAGCGA-3′
p27^{Kip1} rev: 5′-CTCCACAGAACCGGCATTTG-3′
Cyclophilin A for: 5′-GTCAACCCCACCGTGTTCTT-3′
Cyclophilin A rev: 5′-CTGCTGTCTTTGGGACCTTGT-3′

2.9. Statistical analysis

For Western blotting data, the band densities were determined with a UVP bioimaging system (UVP, Upland, CA, USA). The intensity of bands was analyzed using LabWorks Image Analysis software (UVP) and expressed as integrated optical density (IOD). The band intensities were normalized to α-tubulin or β-actin accordingly. All data were presented as mean ± SD and compared using Student's *t*-test.

3. Results

3.1. UBE2M is induced by gemcitabine

To identify gemcitabine (Gem) responsive proteins, the human urothelial carcinoma cell line NTUB1 was treated with or without 0.2 μM Gem and the total proteins were isolated and subjected to two-dimensional gel electrophoresis analysis (Fig. 1A). The images and data were analyzed with background subtraction and volume normalization to adjust for the differences in total protein by the Image Master 2D Platinum software. Nine Gem-induced proteins with a fold increase of between 1.294 and 6.194 were isolated and identified (Table 1). Five of nine proteins are enzymes with important roles in various metabolic pathways including polyamine [spermidine synthase; SRM], cholesterol [phosphomevalonate kinase; PMVK], citric acid cycle [dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex); DLST], heme [uroporphyrinogen decarboxylase; UROD] and lipid metabolism [fatty acid synthase; FASN]. Two proteins, [proteasome (prosome, macropain) subunit, beta type, 6; PSMB6 and ubiquitin-conjugating enzyme E2M; UBE2M], are involved in protein turnover. The biological significance of each protein in Gem action will be of interest. Here, we report our findings on the role of UBE2M.

Further analysis to validate the data obtained from the proteomic approach confirmed that Gem induced UBE2M protein and mRNA levels by 1.79- and 2.79-fold, respectively, in NTUB1 cells (Fig. 1B and C). Furthermore, the induction of UBE2M protein by Gem was time- and dose-dependent (Fig. 1D). These results raise the possibility that UBE2M plays a role in the cellular response to Gem in NTUB1. To further investigate the response of UBE2M to Gem, the level of UBE2M was also examined in the commonly used bladder tumor cell line T24, the prostate cancer cell line PC3, and the pancreatic cancer cell line PANC-1. As showed in Fig. 1E, UBE2M protein was up-regulated by Gem between 1.5

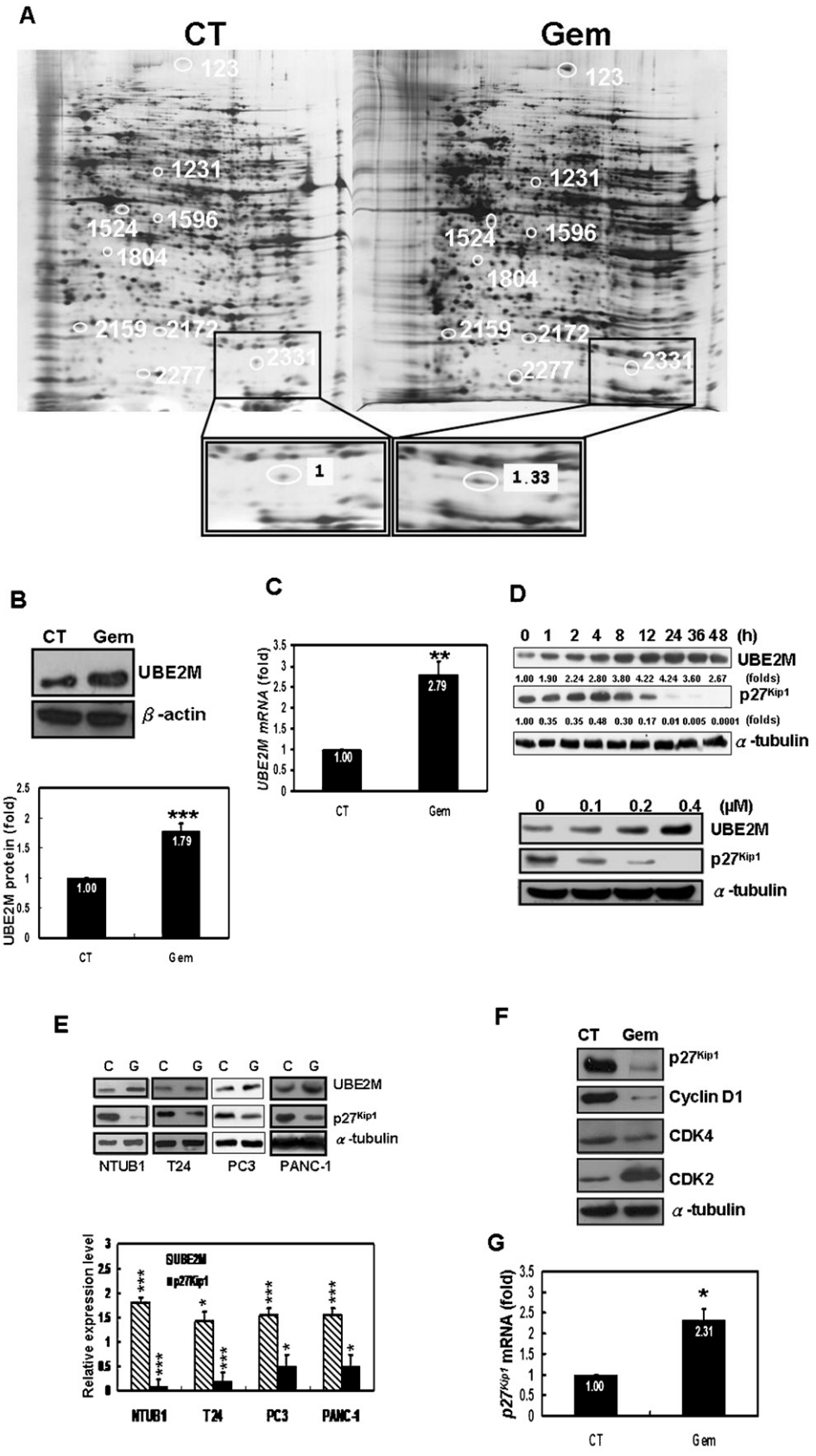

Fig. 1. UBE2M is up-regulated while p27^{Kip1} is down-regulated in gemcitabine-treated cancer cell lines. (A) NTUB1 cells were treated without (CT) or with 0.2 μM gemcitabine (Gem) for 24 h. The cells were harvested and subjected to 2D SDS-PAGE as described in Section 2. The gels were silver stained and analyzed by software to normalize for experimental variations and to identify the indicated proteins as differentially expressed. The region containing spot ID2331 was enlarged and the fold difference is indicated. (B) NTUB1 cells were treated as in panel A and analyzed by Western blotting with UBE2M and β-actin antibodies. Three independent experiments were quantified and expressed as mean ± SD; $^{***}P < 0.005$. (C) NTUB1 cells were treated as in panel (A). RNA was extracted and analyzed by qRT-PCR as described in Section 2. Data represent three independent experiments analyzed in duplicate each (mean ± SD). $^{**}P < 0.01$. (D) NTUB1 cells were treated with 0.2 μM Gem for the indicated times (upper panel)

Table 1
Identification of gemcitabine-induced proteins by two-dimensional gel electrophoresis in NTUB1 cells. Nine Gem-induced spots from Fig. 1A were picked and identified by tandem mass spectrometry. The intensity of individual spot was determined as described in Fig. 1A. The fold difference between spots in the Gem-treated sample (Gem) and the control (CT) is indicated. The gene names and functions are listed according to the annotation in the NCBI databases.

Spot ID	Fold change (Gem/CT)	Gene name	Gene symbol	Accession number	Function
#2159	1.294	Proteasome (prosome, macropain) subunit, beta type, 6	PSMB6	NM_002798.1	Protein degradation
#1804	1.317	Spermidine synthase	SRM	NM_003132.2	Polyamine biosynthesis
#2331	1.331	Ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)	UBE2M	NM_003969.3	Protein degradation
#2172	1.404	Phosphomevalonate kinase	PMVK	NM_006556.3	Cholesterol biosynthesis
#1231	1.577	Dihydrolipoamide S-succinyitransferase (E2 component of 2-oxo-glutarate complex)	DLST	NM_001933.4	Citric acid cycle
#1524	1.581	Stomatin (EPB72)-like 2	STOML2	NM_013442.1	Unknown
#1596	1.619	Uroporphyrinogen decarboxylase	UROD	NM_000374.4	Heme biosynthesis
#2277	3.695	Keratin 9	KRT9	NM_000226.3	Cytoskeleton
#123	6.194	Fatty acid synthase	FASN	NM_004104.4	Fatty acid biosynthesis

and 1.9 fold in these cell lines, supporting the notion that UBE2M may play a role in the cellular response to Gem.

3.2. Induction of UBE2M is associated with reduction of $p27^{Kip1}$ protein levels

Next, we examined the expression of cell cycle-related molecules in response to Gem. Following treatment of NTUB1 cells with 0.2 μM Gem for 24 h, $p27^{Kip1}$, cyclin D1 and CDK4 were down-regulated while CDK2 was highly induced compared to untreated controls (Fig. 1F). While downregulation of cyclin D1 and CDK4 is consistent with previous reports that Gem induces growth arrest [24], loss of $p27^{Kip1}$ expression was surprising. The cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ is a key mediator of cell cycle arrest in the G1 phase and of apoptosis after insults [25] and a known target of the NEDD8 pathway [15]. As seen in Fig. 1D, following treatment of NTUB1 cells with Gem, the level of $p27^{Kip1}$ protein was decreased to undetectable levels by 24 h. The $p27^{Kip1}$ protein was significantly reduced at 0.1 μM Gem and at lowest level with highest dose (Fig. 1D). Similar correlations were also observed in T24, PC3 and PANC1 cells after 24 h of Gem treatment (Fig. 1E). In contrast to the protein, *$p27^{Kip1}$* mRNA level increased 2.31-fold in response to Gem (Fig. 1G), indicating that there are different regulatory mechanisms controlling $p27^{Kip1}$ mRNA and protein expression. Taken together, these results show that UBE2M induction by Gem is associated with downregulation of $p27^{Kip1}$ protein levels, and suggest that regulation at the protein level is particularly important for $p27^{Kip1}$.

3.3. UBE2M augments Gem-induced $p27^{Kip1}$ protein degradation

To delineate whether UBE2M mediates the suppression of $p27^{Kip1}$ by Gem, UBE2M expression was silenced by transfecting gene-specific siRNA into NTUB1 cells. As shown in Fig. 2A, the level of UBE2M protein was dramatically decreased by two different UBE2M-targeted siRNAs (siUBE#1–2) as compared to the mock control (a non-specific control siRNA). By contrast, the level of $p27^{Kip1}$ was substantially increased in UBE2M-depleted cells and remained significantly higher compared to the mock control after 24 h of 0.2 μM Gem treatment (Fig. 2A). Despite UBE2M-silencing, $p27^{Kip1}$ levels were still somewhat reduced after Gem treatment suggesting that alternative pathways to downregulate $p27^{Kip1}$ protein levels may exist. However, these data show that UBE2M plays a significant role in the regulation of $p27^{Kip1}$ protein expression and contributes to the reduction of $p27^{Kip1}$ by Gem.

To clarify if $p27^{Kip1}$ downregulation by Gem was mediated by the proteasome, NTUB1 cells were treated with a combination of 0.2 μM Gem and 1 μM of the proteasome inhibitor MG132. As shown in Fig. 2B, administration of MG132 prevented down-regulation of $p27^{Kip1}$ protein in response to Gem treatment. To further assess the role of UBE2M in Gem-induced proteasome-mediated degradation of $p27^{Kip1}$, NTUB1 cells were sequentially transfected with HA-ubiquitin and UBE2M siRNA followed by treatment with Gem and MG132. Immuno-precipitation of HA-ubiquitin revealed high-molecular weight, poly-ubiquitinated $p27^{Kip1}$ protein species, which were significantly reduced when UBE2M was silenced (Fig. 2C). These results show that UBE2M augments poly-ubiquitination and downregulation of $p27^{Kip1}$ by the proteasome.

3.4. UBE2M-mediated $p27^{Kip1}$ degradation is PI3K-dependent

The phosphoinositide 3-kinase (PI3K)/AKT survival pathway has been extensively studied in Gem action and many studies suggested that PI3K/AKT could be a promising target for combination therapy with Gem treatment [26,27]. PTEN is a major negative regulator of the PI3K/AKT signaling pathway [28]. Consistent with these reports, PTEN activity (by phosphorylation at Ser380) was inhibited following exposure of NTUB1 cells to Gem. PTEN is primarily an inhibitor of PI3K activity, and accordingly phosphorylation of the PI3K substrate AKT at Ser476 was significantly increased, although PI3K protein levels were only slightly induced (Fig. 3A). The PI3K/AKT pathway has been shown to cause $p27^{Kip1}$ degradation [29,30]. Therefore, we next investigated if PI3K promotes UBE2M expression, which may mediate $p27^{Kip1}$ protein degradation. To test this, the PI3K specific inhibitor LY294002 was applied to Gem-treated NTUB1 cells simultaneously for 24 h. As shown in Fig. 3B, LY294002 alone reduced basal UBE2M expression and completely prevented its induction by Gem. Furthermore, LY294002 rescued the expression of $p27^{Kip1}$ protein in the presence of Gem. Given that LY294002 alone did not lead to a significant increase in $p27^{Kip1}$ protein levels despite reduced UBE2M, we speculate that Gem promotes $p27^{Kip1}$

or with the indicated dosages for 24 h (lower panel). Western analysis was performed with UBE2M, $p27^{Kip1}$ and α-tubulin antibodies. Numbers indicate the densitometric values after normalization to the loading control and relative to untreated samples. (E) Representative Western analysis of the indicated cell lines treated without (C) or with (G) 0.2 μM Gem (NTUB1, T24) and 2 μM Gem (PC3, PANC-1) for 24 h and analyzed as above. Gem concentrations were chosen based on varying IC_{50} values (~0.01 μM for T24 and ~0.3 μM for PC3 and PANC-1 cells; data not shown). The graph shows densitometric quantification of UBE2M and $p27^{Kip1}$ expression (mean ± SD) in Gem treated cells from three independent experiments relative to respective untreated control; $^{*}P < 0.05$ and $^{***}P < 0.005$. (F) NTUB1 cells were seeded and the next day treated without (CT) or with 0.2 μM gemcitabine (Gem) for 24 h followed by Western analysis of whole cell extracts with the indicated antibodies. (G) The cDNAs used in panel C were analyzed for *$p27^{Kip1}$* mRNA expression by real-time qRT-PCR as described; $^{*}P < 0.05$.

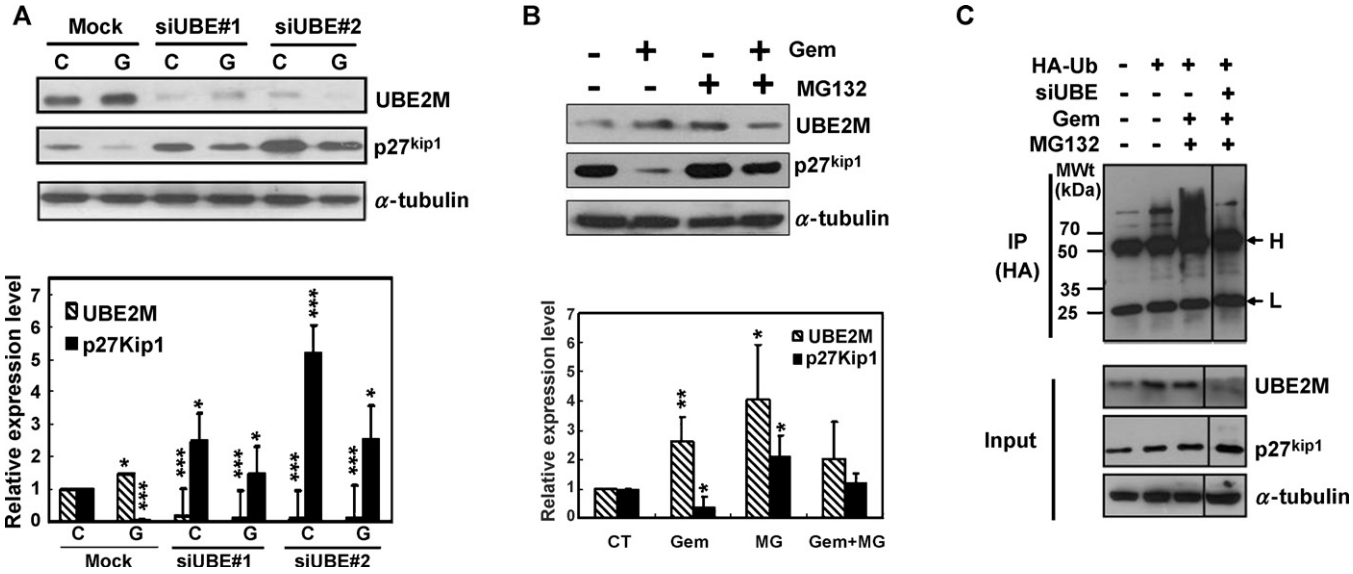

Fig. 2. Silencing of UBE2M expression prevents Gem-mediated $p27^{Kip1}$ downregulation, which is proteasome-dependent. (A) NTUB1 cells were transiently transfected with 50 nM of non-specific siRNA control (Mock) or two individual UBE2M -targeted siRNAs (siUBE#1 and 2) and treated without (C) or with 0.2 μM gemcitabine (G) for 24 h. Western analysis was performed as described in Fig. 1D. The graph shows densitometric quantification of UBE2M and $p27^{Kip1}$ expression (mean ± SD) from three independent experiments relative to respective untreated control; $^{*}P < 0.05$ and $^{***}P < 0.005$. (B) NTUB1 cells were treated with 0.2 μM Gem and/or 1 μM MG132 for 24 h and harvested for Western analysis with UBE2M, $p27^{Kip1}$ and α-tubulin antibodies, respectively. The graph shows densitometric quantification of UBE2M and $p27^{Kip1}$ expression (mean ± SD) from three independent experiments relative to untreated controls; $^{*}P < 0.05$ and $^{**}P < 0.01$. (C) NTUB1 cells were transfected with HA-ubiquitin expression construct followed by UBE2M-targeted siRNA (siUBE#2) and the indicated treatments for 24 h. Western blots of immunoprecipitates with $p27^{Kip1}$ antibody and a fraction of the input samples were analyzed with the indicated antibodies. The samples were analyzed on the same gel with intermediate lanes deleted as indicated by the line. The arrows indicate antibody reaction with IgG (H and L).

degradation not only through UBE2M expression but also by additional signals, which may regulate the activity of proteins in the ubiquitination/degradation pathway. However, our results show that PI3K activity is necessary for UBE2M expression and for $p27^{Kip1}$ protein degradation in response to Gem, which further corroborates the link between UBE2M and $p27^{Kip1}$ downregulation by Gem.

3.5. Depletion of UBE2M increases Gem resistance

To determine the role of UBE2M in Gem sensitivity, the expression of UBE2M was silenced in NTUB1 cells by transfection of specific siRNA against UBE2M (siUBE) as characterized in Fig. 2A.

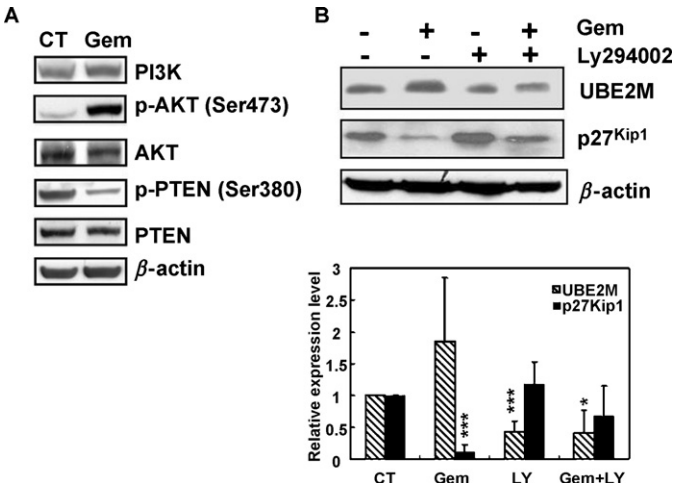

Fig. 3. UBE2M expression is PI3K-dependent. (A) NTUB1 cells were treated without (CT) or with 0.2 μM gemcitabine (Gem) for 24 h and harvested for Western analysis with the indicated antibodies. (B) NTUB1 cells were treated with 0.2 μM Gem and/or 10 μM LY294002 for 24 h and harvested for Western blotting with UBE2M, $p27^{Kip1}$ and β-actin antibodies. The graph shows densitometric quantification of UBE2M and $p27^{Kip1}$ expression (mean ± SD) from three independent experiments relative to respective untreated controls; $^{*}P < 0.05$ and $^{***}P < 0.005$.

Indeed, suppression of UBE2M rendered NTUB1 cells more resistant to Gem (Fig. 4). The IC_{50} values significantly increased from 6.97 nM (mock) to 11.07 and 16.63 nM in UBE2M-depleted cells (siUBE#1 and 2), respectively (Fig. 4B).

To further address the contribution of UBE2M to Gem resistance, three NTUB1-derived Gem-resistant sublines (NG0.6, NG0.8 and NG1.0) were established in our laboratory by chronic exposure of cells to increasing concentrations of Gem. The IC_{50} values were then determined by MTT assay. As shown in Fig. 5A, the sublines exhibited IC_{50} values of 10.31 ± 1.23 μM (NG0.6), 12.67 ± 1.61 μM (NG0.8) and 14.59 ± 1.82 μM (NG1.0), respectively, while the IC_{50} of the parental NTUB1 cells was only 7.68 ± 1.1 nM. Thus, the ratio of the IC_{50} increased 1342-, 1650-, and 1900-fold for each of the Gem-resistant sublines (NG0.6, NG0.8 and NG1.0) compared to the parental NTUB1 cells. Notably, the level of UBE2M protein expression gradually decreased while $p27^{Kip1}$ levels increased as NTUB1 cells had acquired more resistance to Gem (Fig. 5B). Three PANC-1 derived Gem-resistant sublines (PG100, PG300 and PG1500) were also established and further evaluated. Once again, inverse expression levels of UBE2M and $p27^{Kip1}$ were observed with Gem-resistance in these pancreatic cancer cell sublines (Fig. 5B). These data show that acquisition of Gem-resistance is accompanied by the loss of a pathway that mediates Gem cytotoxicity, i.e. induction of UBE2M and downregulation of $p27^{Kip1}$.

4. Discussion

Gemcitabine (Gem) is the first-line chemotherapeutic drug for metastatic bladder cancer and pancreatic adenocarcinoma [31,32]. Despite its relative efficacy, the mode of action is still poorly characterized. In addition, the molecular mechanisms that lead to Gem resistance in patients also need further characterization. In this report, we show that UBE2M mediates $p27^{Kip1}$ downregulation in response to Gem and promotes Gem cytotoxicity (Fig. 6). To our knowledge, the present study is the first to demonstrate an association of UBE2M and Gem responsiveness in tumor cells. Furthermore, the identification of this novel link of UBE2M to Gem

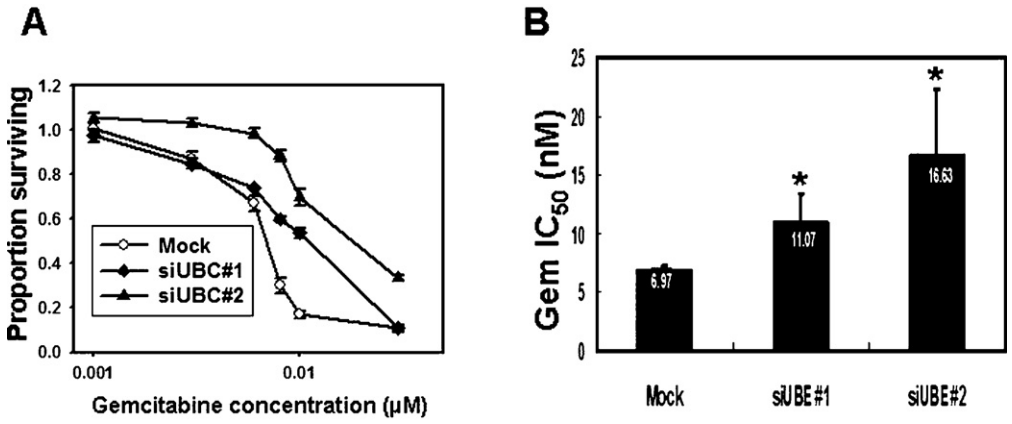

Fig. 4. Silencing of UBE2M expression reduces Gem sensitivity in NTUB1 cells. (A) NTUB1 cells were treated as described in Fig. 2A with increasing concentrations of Gem and analyzed by MTT cytotoxicity assay as described in Section 2. (B) The IC_{50} values were calculated by the Calcusyn program from three independent experiments. The data are expressed relative to the Mock control (mean ± SD); $^{*}P < 0.05$.

cytotoxicity and of Gem action to $p27^{Kip1}$ degradation may have important implications for cancer treatment as discussed below.

UBE2M is an E2 NEDD8-conjugating enzyme and NEDD8-conjugation plays an important role in tumorigenesis and is being explored as a target for cancer therapy [33,34]. UBE2M mRNA expression is elevated by ionized iodization in human esophageal cancer cell lines but its role and significance in the response had not been addressed [35]. We have shown that UBE2M was induced by Gem and that UBE2M contributed to Gem cytotoxicity. Our results demonstrate that targeting the NEDD8 pathway can also interfere with drug response. Therefore, further studies are warranted to better define which cancer types or specific tumors may benefit from NEDD8-targeted therapeutics.

Cell cycle dis-regulation is a hallmark of cancer. $p27^{Kip1}$ is a key regulator of the G1 to S phase progression and is mainly regulated through ubiquitin-dependent proteasomal degradation involving the SCF^{Skp2} polyubiquitination complex [14]. UBE2M and the NEDD8 pathway have been shown to enhance the ubiquitin-dependent degradation of $p27^{Kip1}$ [15]. Downregulation of the $p27^{Kip1}$ protein is observed in many cancer cell types and $p27^{Kip1}$

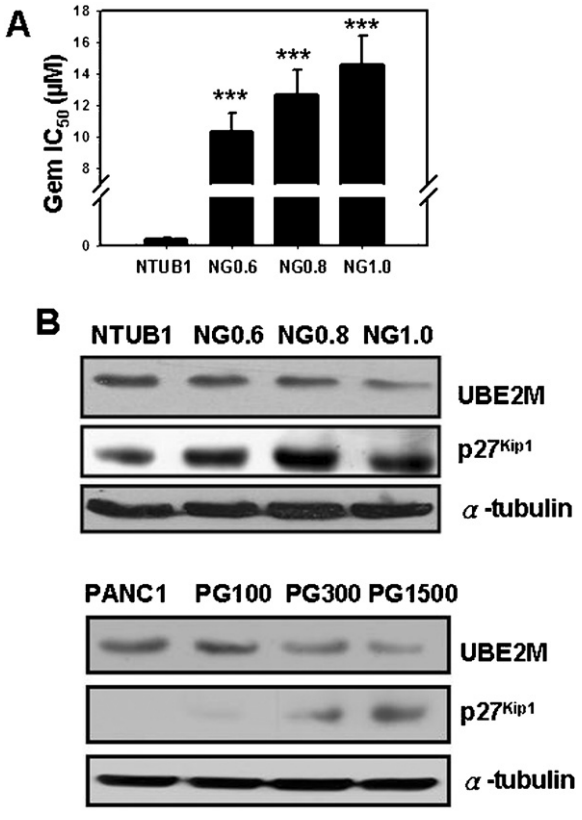

Fig. 5. Inverse correlation of UBE2M expression levels with gemcitabine resistance and $p27^{Kip1}$ expression. (A) NTUB1 and three NTUB1-derived gemcitabine-resistant sublines (NG0.6, NG0.8 and NG1.0) were analyzed for gemcitabine sensitivity as described in Fig. 4B, $^{***}P < 0.005$. (B) Western analysis of protein lysates from NTUB1 and three NTUB1-derived gemcitabine resistant sublines (NG0.6, NG0.8 and NG1.0) and PANC1 and three PANC1-derived gemcitabine resistant sublines (PG100, PG300 and PG1500) with UBE2M, $p27^{Kip1}$ and α-tubulin antibodies.

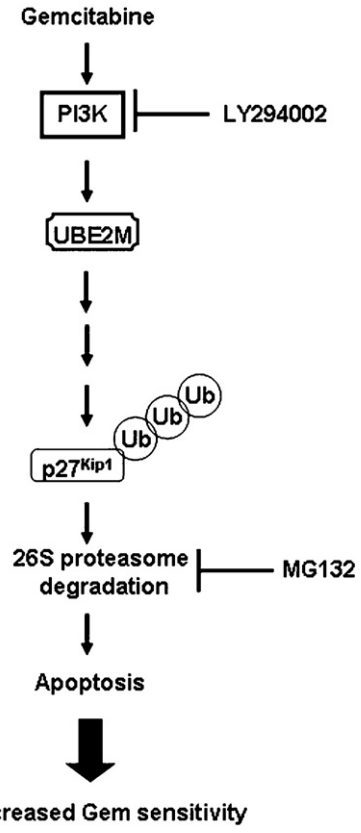

Fig. 6. Proposed model for UBE2M-mediated gemcitabine cytotoxicity. Gemcitabine activates UBE2M through a pathway that requires PI3K activity. UBE2M promotes ubiquitin (Ub)-dependent proteasomal degradation of $p27^{Kip1}$. This results in increased apoptosis and Gem cytotoxicity.

expression is associated with enhanced sensitivity to certain therapeutic drugs, indicating its role as a tumor suppressor [13]. On the other hand, it has been reported that $p27^{Kip1}$ can be pro-oncogenic by enhancing cell motility through RhoA activation [37], and $p27^{Kip1}$ can be associated with paclitaxel- or cisplatin-resistance [36,37]. In the current study, Gem-induced $p27^{Kip1}$ reduction was observed in four different cancer cell lines, while increased $p27^{Kip1}$ protein levels and decreased UBE2M levels were associated with acquired drug resistance of bladder cancer and pancreatic cancer cell lines. To our knowledge, this is the first report of $p27^{Kip1}$ overexpression being associated with Gem-resistance, which supports a potential pro-oncogenic function of $p27^{Kip1}$. The CDK inhibitor $p21^{Cip1}$ has been linked to drug resistance because it augments cell cycle arrest, which allows cells to repair damaged DNA [37]. A similar mechanism can be envisioned for $p27^{Kip1}$ and warrants further investigations into the role of $p27^{Kip1}$ in the response to Gem.

We observed that Gem activated the PI3K/AKT pathway and that inhibition of PI3K by LY294002 prevented Gem-induced UBE2M expression and $p27^{Kip1}$ downregulation. These data are consistent with the reported role of the PI3K/AKT pathway in promoting $p27^{Kip1}$ degradation [38]. However, PI3K/AKT activation is also known to augment survival in Gem treated cells [26]. Our results suggest that PI3K/AKT also additionally contributes to the cytotoxic effect of Gem. Hence, targeting the PI3K/AKT pathway in combination with Gem may also interfere with the cytotoxic efficacy of Gem.

In summary, our data provide new insights into the molecular responses to Gem by identifying UBE2M as a mediator of Gem cytotoxicity and by demonstrating that $p27^{Kip1}$ is downregulated by gemcitabine through UBE2M, with potentially important implications for mechanisms of drug resistance.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgements

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