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Role of eIF3a in regulating cisplatin sensitivity and nucleotide excision repair of nasopharyngeal carcinomas

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Abstract

Translational control at the initiation step has been recognized as a major and important regulatory mechanism of gene expression. eIF3a, a putative subunit of eIF3 complex, has recently been shown to play an important role in regulating translation of a subset of mRNAs and found to correlate with prognosis of cancers. In this study, using nasopharyngeal carcinoma (NPC) cells as a model system we tested the hypothesis that eIF3a negatively regulates synthesis of nucleotide excision repair (NER) proteins and, thus, NER activities and cellular response to treatments with DNA damaging agents such as cisplatin. We found that a cisplatin-sensitive subclone S16 isolated from a NPC cell line CNE2 via limited dilution has increased eIF3a expression. Knocking down its expression in S16 cells increased cellular resistance to cisplatin, NER activity, and synthesis of NER proteins XPA, XPC, RAD23B, and RPA32. Altering eIF3a expression also changed cellular response to cisplatin and UV treatment in other NPC cell lines. Taken together, we conclude that eIF3a plays an important role in cisplatin response and NER activity of nasopharyngeal carcinomas by suppressing synthesis of NER proteins.

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Keywords

cisplatin sensitivity; eIF3a; nasopharyngeal carcinoma; nucleotide excision repair; translational control

Introduction

Translational control plays a major role in regulating gene expression and occurs primarily at the initiation step involving multiple eukaryotic translation initiation factors (eIFs) (Dong and Zhang, 2006; Mathews *et al.*, 2000). eIF3a, the largest subunit of eIF3 complex, has been suggested to play roles in regulating translation of a subset of mRNAs and in regulating cell cycle progression and cell proliferation (Dong *et al.*, 2009a; Dong and Zhang, 2006). Suppressing endogenous eIF3a expression has been shown to reverse malignant phenotype of human cancer cells (Dong *et al.*, 2004) while over-expression of ectopic eIF3a has been shown to promote malignant transformation of mammalian cells (Zhang *et al.*, 2007). eIF3a expression has also been correlated with better prognosis of human cancer patients and eIF3a up-regulation in lung cancer patients correlates with their response to platinum-based chemotherapy and contributes to increased cisplatin sensitivity (Yin *et al.*, 2010). These observations suggest that eIF3a plays an important role in tumorigenesis and in cancer cell response to chemotherapeutics, possibly by regulating gene expression.

Nasopharyngeal carcinoma (NPC) has a high incidence in South China although it is a rare disease in other parts of the world. The first treatment choice for NPC is radiation therapy, which has given satisfactory outcomes in early stage of NPC (Xiao *et al.*, 2009). However, approximately 70% of patients with newly diagnosed NPC present locally advanced, non-metastatic stage III or IV diseases (Agulnik and Siu, 2005) and experience poor prognosis from radiation therapy with a 5-year overall survival rate varying from 37%–61% (Afqir *et al.*, 2009; Agulnik and Siu, 2005). Concurrent chemo-radiotherapy is now considered as a standard treatment approach for locally advanced NPC and cisplatin-based regimen has been identified as the best protocol by meta-analysis (Baujat *et al.*, 2006; Thephamongkhol *et al.*, 2004). Compared with radiotherapy alone, combination of cisplatin-based chemotherapy with radiation improved 5-year overall survival by 6% (from 56% to 62%) and 5-year disease-free survival by 10% (from 42% to 52%) for locally advanced NPC (Baujat *et al.*, 2006). Unfortunately, many NPC patients do not benefit much from the concurrent chemo-radiotherapy, possibly due to variation in treatment responses and cisplatin resistance. Clearly, finding the underlying mechanisms of chemosensitivity/resistance in NPC will likely help predict potential responses of NPC patients for better clinical outcome with less toxicity to the patients.

Toward this goal, we have selected a cisplatin sensitive S16 clone from an NPC cell line via limited dilution and demonstrated that eIF3a expression is increased and contributes to cisplatin sensitivity in S16 clone. Knocking down eIF3a expression in S16 cells up-regulates cellular nucleotide excision repair (NER) activity via increasing the synthesis rate of NER proteins such as XPA, XPC, RAD23B, and RPA. These observations suggest that eIF3a may be a major candidate contributing to cisplatin sensitivity of NPC and implicates that eIF3a

may be used as a biomarker predicting treatment outcome of cisplatin-based chemotherapy for locally advanced NPC.

Results

Selection and characterization of a cisplatin-sensitive clone S16 from NPC CNE-2 cells

Previously, stepwise selections with anticancer drugs have been used as a standard method to create model cell lines for laboratory studies of acquired drug resistance. However, tumor heterogeneity could be a natural intrinsic cause of resistance (or sensitivity) in response to drug treatment. To investigate the mechanism of cisplatin sensitivity/resistance of NPC cells, we performed a study using an approach, different from the standard stepwise drug selection, by clonal selection using limited dilution. With this approach, a cisplatin-sensitive clone, S16, was selected from the parental CNE-2 cell line. S16 is about 3-fold more sensitive to cisplatin than the parental CNE-2 cells with IC_{50} 2.5±0.31 μM for S16 *vs* 8.7±0.75 μM for CNE-2 (Fig. 1A).

To verify that the S16 clone is authentically originated from CNE-2, we examined their p53 mutation and short tandem repeat (STR) profiles. Both S16 and CNE-2 harbor an identical point mutation at codon 280 (exon 8) of p53 with a change of AGA (arginine) to ACA (threonine). The short tandem repeat (STR) profiles of CNE-2 and S16 cells are also the same, except lost allele 216 at D21S11 locus for S16 cells (supplemental Table S1), showing a spontaneous genetic alteration which can be common in cancer cell lines. These findings suggest that S16 is likely derived from CNE2 but with an increased sensitivity to cisplatin.

To determine if the increased cisplatin sensitivity of S16 cells is due to increases in cisplatin-induced apoptosis, S16 and its parental cell line CNE-2 were treated with cisplatin at 8.7 μM for 24 and 48 hrs followed by DAPI staining analysis of disintegrated nuclei, an indicator of apoptosis. As shown in Fig. 1B and 1C, S16 cells have significantly more cells with disintegrated nuclei compared with CNE-2 cells at both 24 and 48 hrs of cisplatin treatment. The cisplatin-induced apoptosis can be reversed by pretreatment with z-VAD-fmk, a broad-spectrum caspase inhibitor. These observations suggest that S16 cells are more sensitive to cisplatin-induced apoptosis than its parental CNE-2 cells.

Contribution of eIF3a over-expression to cisplatin sensitivity of S16 cells

Previously, it has been shown that cervical and esophageal cancer patients with high eIF3a level have better relapse-free and overall survival than those with low eIF3a expression (Chen and Burger, 1999; Dellas *et al.*, 1998). Since cisplatin is a primary anti-cancer drug for treating these cancers, it is conceivable that eIF3a may contribute to cisplatin response of these cancers. Indeed, it was recently found that eIF3a up-regulation in lung cancer patients correlated with their response to platinum-based chemotherapy and contributed to increased cisplatin sensitivity (Yin *et al.*, 2010). Based on these previous observations, we propose that eIF3a expression may be up-regulated in S16 cells compared with CNE-2 and contributes to the increased cisplatin sensitivity of S16 cells.

To test this hypothesis, we first examined eIF3a expression level in S16 and CNE-2 cells using Western blot. As shown in Fig. 2A, S16 cells clearly have more eIF3a than CNE-2

cells. We next examined if the up-regulated eIF3a expression possibly contributes to the increased cisplatin sensitivity of S16 cells by knocking down eIF3a expression in S16 cells followed by MTT assay. As shown in Fig. 2B, eIF3a is successfully knocked down as determined using Western blot. S16 cells with reduced eIF3a expression are also more resistant to cisplatin than the control cells transfected with scrambled siRNA (Fig. 2B) with ~3.5-fold increase in relative resistance factor as determined using MTT assay (Fig. 2D). Similar results were also observed using colony formation assay (data not shown), eliminating the possible contribution of cell proliferation on drug sensitivity. Thus, the up-regulated eIF3a level likely contributes to the increased cisplatin sensitivity of S16 cells compared to its parental CNE-2 cells.

To confirm the above conclusion, we next performed a reverse experiment by increasing eIF3a expression in the parental CNE-2 cells and determined if the increased eIF3a level would cause increased cisplatin sensitivity in CNE-2 cells. For this purpose, a pool of stable CNE-2 cells transfected with eIF3a cDNA for ectopic eIF3a over-expression was selected and subjected to MTT assay (Fig. 2C). It was found that the CNE-2 cells with stable eIF3a over-expression were more sensitive to cisplatin with a 2-fold decrease in relative resistance factor (RRF) compared to the control cells transfected with vector (Fig. 2D). Taken together, we conclude that eIF3a over-expression contributes to the increased cisplatin sensitivity of S16 cells.

The contribution of eIF3a to cisplatin sensitivity is not cell line specific

To rule out the possibility that the contribution of eIF3a to cisplatin sensitivity is specific to CNE-2 and its derivative cell lines, we next tested another NPC cell line, SUNE-1, by knocking down eIF3a expression followed by MTT assay. For this experiment we also included the parental CNE-2 cell line to determine if knocking down eIF3a expression would further increase its cisplatin resistance. As shown in Fig. 3A, eIF3a expression is successfully knocked down by siRNA in both cell lines as determined using Western blot. Both cell lines with reduced eIF3a level are also more resistant to cisplatin (Fig. 3B) with ~2-fold increase in RRF compared to their respective control cells transfected with scrambled siRNA (Fig. 3C). Thus, the role of eIF3a in cisplatin sensitivity is likely not cell line specific and decreasing its level in the parental resistant CNE-2 cells could further increase its cisplatin resistance.

The up-regulated eIF3a level contributes to the increase in cisplatin-induced apoptosis of S16 cells

As discussed above, S16 cells have an increased sensitivity to cisplatin-induced apoptosis compared to its parental CNE-2 cells (Fig. 1). To determine if eIF3a up-regulation mediates this process, we first examined if knocking down eIF3a level in S16 cells would reduce cisplatin-induced apoptosis by analysis of disintegrated nuclei as described above. As shown in Fig. 4A, S16 cells transfected with eIF3a siRNA clearly have significantly less apoptosis following cisplatin treatment than the control cells transfected with scrambled siRNA. We next examined cleavage of PARP, a 115-kDa protein substrate of caspases during execution of apoptosis. Cleavage of PARP by caspases yields an 85-kDa fragment. As shown in Fig. 4B, S16 cells transfected with eIF3a siRNA generate much less cleaved 85-kDa PARP

product than the control cells following cisplatin treatment. Thus, the up-regulated eIF3a level in S16 cells likely mediates the increase in cisplatin-induced apoptosis of S16 cells compared to its parental CNE2 cells.

eIF3a down-regulates NER activity and expression of NER proteins

Cisplatin causes cytotoxicity by causing DNA damages with formation of cisplatin-DNA adducts. To survive this DNA damage, cells use mainly nucleotide excision repair (NER) to remove cisplatin-DNA adducts. Thus, we hypothesize that eIF3a expression may reduce NER activities, which in turn increases cisplatin sensitivity and cisplatin-induced apoptosis. To test this hypothesis, we first examined if eIF3a expression affects cellular response to UV treatment which causes DNA damages that are also repaired by NER. For this purpose, S16 cells transfected with eIF3a or scrambled control siRNA were subjected to UV treatment followed by analysis of apoptosis using ELISA. As shown in Fig. 4C, S16 cells transfected with eIF3a siRNA are significantly more resistant to UV-induced apoptosis than the control cells at both 30 and 60 J/cm^2.

Next, the effect of knocking down eIF3a expression in S16 cells on NER activity was determined using host cell reactivation (HCR) assay (Qiao *et al.*, 2002). For this purpose, a plasmid containing luciferase reporter was first UV-irradiated to generate DNA adducts. The damaged reporter plasmids were then transfected into S16 cells with reduced eIF3a expression followed by analysis of luciferase activity with the assumption that the damaged reporter plasmid will give the expression of luciferase only after its UV-induced damages are repaired by the cellular NER mechanism (Qiao *et al.*, 2002). As shown in Fig. 5A, knocking down eIF3a expression significantly increases NER activities by ~1.5 fold. Thus, the up-regulated eIF3a expression in S16 cells likely suppresses cellular NER activity, which in turn contributes to the increased sensitivity to cisplatin or UV-induced apoptosis.

NER represents one of the known major DNA repair mechanisms and it involves several key DNA repair proteins (Sancar *et al.*, 2004; Zou *et al.*, 2006). Because eIF3a has been shown previously to play important roles in regulating gene expression by controlling protein synthesis (Dong and Zhang, 2006; Yin *et al.*, 2011), we hypothesize that the increased eIF3a level in S16 cells likely down-regulates the synthesis of NER proteins which in turn down-regulates NER activity. To test this hypothesis, we first determined the effect of eIF3a on the expression of NER proteins following eIF3a knockdown using Western blot analysis. As shown in Fig. 5B, knocking down eIF3a expression clearly increases the level of key NER proteins XPA, XPC, RPA32, and RAD23B. However, the mRNA level of these NER proteins is not affected by knocking down eIF3a expression (Fig. 5C). Thus, the effect of eIF3a on the expression of NER proteins is likely at their protein levels.

To further determine if eIF3a possibly regulates the synthesis but not stability of these representative NER proteins, we performed pulse and pulse-chase labeling in combination with immunoprecipitation and autoradiography to measure the synthesis and half life of these NER proteins. As shown in Fig. 6, eIF3a knockdown in S16 cells has no effect on the half-life of these NER proteins but dramatically increases their synthesis rate as determined using pulse-chase and pulse labeling experiments, respectively. Taken together, these

findings suggest that eIF3a negatively regulates the synthesis of NER proteins by suppressing the translation of their mRNAs without affecting their transcription to produce mRNAs or stability (or degradation) of these proteins. It is noteworthy that the degradation of these NER proteins may occur differently with XPA and XPC in bi-phase while RPA32 and RAD23B in mono-phase mode. Regardless, eIF3a does not appear to have any effect on their degradation.

Discussions

In this study, we have successfully selected a cisplatin sensitive NPC cell clone, S16, from CNE-2 cell line by limited dilution and demonstrated that eIF3a over-expression contributes to the increased cisplatin sensitivity in S16 cells by suppressing synthesis of NER proteins which, in turn, contributes to the increase in cisplatin-induced apoptosis and cisplatin sensitivity. This outcome establishes eIF3a as a potential marker predicting cisplatin responses and helping guide future treatment of NPC patients.

In this study, we have successfully generated a cisplatin sensitive clone, S16, from a NPC cell line CNE-2 by limited dilution. S16 appears to be generated authentically from CNE-2 possibly due to spontaneous genetic changes. This argument is supported by the finding of only a partial incompatibility at D21S11 locus between S16 and its parental CNE-2 cells. The finding of increased eIF3a expression also supports the above argument. However, it is currently unknown how eIF3a expression is up-regulated in S16 cells. Previously, increased expression of eIF3a has been found in various human cancers compared to normal tissues (Bachmann *et al.*, 1997; Chen and Burger, 1999; Chen and Burger, 2004; Dellas *et al.*, 1998; Pincheira *et al.*, 2001). It is possible that the increased eIF3a expression in these systems uses similar mechanisms of up-regulation. Future studies investigating the mechanism of eIF3a expression regulation will be beneficial to address this issue.

eIF3a was initially thought to be essential for global translation initiation process. However, accumulating evidences suggest that eIF3a may play a more important regulatory role in mRNA translation. It was first found that the eIF3 complex lacking eIF3a worked without distinction from eIF3 complex containing eIF3a in forming pre-initiation complexes (Chaudhuri *et al.*, 1997). Later, it was found that knocking down eIF3a expression did not severely suppress global mRNA translation but, in fact, it increased synthesis of proteins such as p27 while suppressing synthesis of ribonucleotide reductase M2 (Dong *et al.*, 2004). The finding in this paper that knocking down eIF3a increased the synthesis of NER proteins further enforces the possible regulatory role of eIF3a in translational control. Furthermore, we have also observed previously that knocking down or ectopically over-expressing eIF3a does not affect the expression of other putative eIF3 subunits, suggesting that the regulatory role of eIF3a may not involve the change in expression of other putative eIF3 subunits (Dong *et al.*, 2004).

It is still not yet understood how eIF3a participates in translational control. The finding that eIF3a possibly regulates the synthesis of a group of proteins in NER is also puzzling. Nevertheless, it has been suggested previously that the 5′- and 3′-UTRs of mRNAs under eIF3a regulation may mediate this regulatory process. For example, the 5′- and 3′-UTRs of

p27 and ribonucleotide reductase M2 appeared to be responsible for eIF3a regulation of the translation of these genes (Dong *et al.*, 2004). Recently, it was found that the synthesis of phosphatase of regenerating liver 3 (PRL-3) was suppressed by PolyC-RNA-binding protein 1 (PCBP1) via PCBP1 binding to a triple GCCCAG motifs in the 5′-UTR of PRL-3 mRNAs (Wang *et al.*). Thus, it is possible that the mRNAs under eIF3a regulation may have a consensus sequence motif in their UTRs that can possibly bind to eIF3a. This possibility is waiting to be tested.

Cisplatin is an anticancer drug used to treat many types of cancers including nasopharyngeal, lung, ovarian, cervical, and esophageal cancers. Unfortunately, lack of response to cisplatin frequently occurs, which limits successes in the treatment of these cancers. Increase in DNA damage repair is one of the mechanisms of cisplatin resistance (Siddik, 2003). NER is the main pathway for the repair of cisplatin-induced DNA damage (Neher *et al.*, 2010a; Neher *et al.*, 2010b; Nouspikel, 2009), which functions by recognizing DNA adducts via XPC-RAD23B subcomplex and forming NER complex through the binding of XPA, RPA, and other proteins (Friedberg, 2001). NER pathway defect results in cellular hypersensitivity to cisplatin, and restoring NER activity will increase cellular resistance (Furuta *et al.*, 2002; Siddik, 2003). The current finding that eIF3a expression negatively regulates the expression of NER-related proteins (XPA, XPC, RAD23B and RPA) and contributes to cisplatin sensitivity suggests that assessing eIF3a level may help predict cisplatin responses and design individualized treatment strategies for patients with these cancers. Previously, it has been observed that cervical and esophageal cancer patients with high eIF3a level had better relapse-free and overall survival than that with low eIF3a expression (Chen and Burger, 1999; Dellas *et al.*, 1998). Since cisplatin is a primary anti-cancer drug for treating these cancers, it is conceivable that eIF3a may contribute to cisplatin response in these cancer patients. It is also tempting to speculate that eIF3a expression may contribute to cellular response to cisplatin in any cancers that are treated with this drug and that assessing eIF3a level may help predict response and design treatment strategies for these cancers.

Materials and Methods

Materials

Rabbit polyclonal IgGs against XPA, XPC, RPA32, RAD23B, as well as eIF3a siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa cruz, CA, USA). Scrambled control siRNA (Silencer Negative Control #1 siRNA) was purchased from Applied Biosystems Ambion (Austin, TX, USA). AmpliTaq Gold polymerase and Power SYBR® Green RNA-to-CT™ 1-Step Kit were from Applied Biosystems Life Technologies Corp. (Carlsbad, CA, USA). Antibody against cleaved PARP (19F4, 9546) was from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody against actin, HRP-conjugated anti-mouse or rabbit secondary antibodies, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and cis-Dichlorodiammine platinum(II) (cisplatin) were from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), G418, Hoechst 33342, Superscript™ II reverse transcriptase, and Lipofectamine™ 2000 were from Invitrogen (Carlsbad, CA, USA). The enhanced chemiluminescence (ECL) system and

[^{35}S]Methionine were from Amersham Biosciences (Piscataway, NJ, USA) and PerkinElmer Life Sciences (Boston, MA, USA), respectively. Polyvinylidene difluoride (PVDF) membrane and concentrated protein assay dye reagents were from Bio-Rad (Hercules, CA, USA). All other reagents were of molecular biology grade and obtained from Sigma-Aldrich or Fisher Scientific (Chicago, IL, USA).

Cell lines, characterization, and transfection

Human nasopharyngeal carcinoma cell lines CNE-2 and SUNE-1 were maintained in DMEM containing 10% fetal bovine serum and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. S16, a cisplatin-sensitive subclone derived from CNE-2, was isolated by limited dilution as previously described (Qian *et al.*, 2006) and was maintained similarly as the parental CNE-2 cells.

For short tandem repeat (STR) profiles analysis, six STR loci: HUMTHO1, HUMvWA, D18S51, D20S85, D21S11, and HPRTB were examined. The primers for the first five STR loci were designed according to previous report (Oldroyd *et al.*, 1995), with the forward primers labeled with FAM (for HUMTHO1, HUMVWFA31/A, D18S51, and D21S11 loci) or HEX (for D20S85 locus). HPRTB, a tetranucleotide repeat at chromosome Xq26.1, was amplified with FAM-labeled forward primer (5′-FAM-ATGCCACAGATAATACACATCCCC-3′) and unlabeled reverse primer (5′-CTCTCCAGAATAGTTAGATGTAGG-3′). PCR was performed using AmpliTaq Gold polymerase. One microliter of each PCR product was added to 5 μl of DNase-free, RNase-free distilled water, 10 μl of Hi-Di formamide and 0.2 μl of ROX 400HD size standard, and denatured at 95o C for 5 min before loading the samples into an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Analysis of raw data was performed with Genescan v3.7 software (Applied Biosystems). For p53 mutation analysis, 1 μg of cellular total RNA was reverse-transcribed into cDNA with Superscript™ II reverse transcriptase. p53 full-length cDNA was obtained by consequent PCR and sequenced for mutant analysis.

For transient transfection, cells were seeded in 10-cm dish and cultured for 24 hrs prior to transfection with eIF3a or scrambled control siRNAs (200~400 pmol/dish) using Lipofectamine™ 2000 transfection reagent according to the manufacture's instruction. The cells were cultured for additional 24 hrs and were harvested for further analysis. To generate stable CNE-2 cells with over-expression of ectopic eIF3a, CNE-2 cells were seeded in 6-cm dish and culture for 24 hrs, and then were transfected with eIF3a expression plasmid pCβA-p170 (Dong and Zhang, 2003) or blank vector pCβA. Twenty-four hours after transfection, the standard media were replaced with media containing G418. Stable transfected cell lines were obtained by selecting in media containing 750 μg/mL of G418 for 3 weeks, and then maintained in media containing 200μg/mL of G418.

Western blot analysis

Western blot analysis was performed as described previously (Dong *et al.*, 2009b). Briefly, cells were harvested and lysed with TNN-SDS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 2 mM PMSF, 0.5% NP-40, and 0.1% SDS) at 4°C for 30 min followed by centrifugation (10,000 g for 10 min at 4°C) to

remove cell debris. Supernatants were collected and protein concentrations were measured followed by separation using SDS-PAGE. The proteins were then transferred to PVDF membranes which were probed with specific primary antibodies, followed by reaction with relevant HRP-conjugated secondary antibodies and detecting signals with ECL system.

Real-time RT-PCR analysis

Real-time RT-PCR was performed as previously described (Dong *et al.*, 2005). Briefly, cells were harvested and total RNAs were extracted using RNeasy Mini Kit (Qiagene, Valencia, CA, USA) followed by real time RT-PCR using Power SYBR® Green RNA-to-CT™ 1-Step Kit and primers for each gene as listed in supplemental Table S2. The threshold cycle (Ct) were determined and normalized against that of GAPDH internal control. The relative mRNA levels were shown as the value of $2^{\Delta Ct}$. The changes in mRNA levels were presented as a ratio of relative mRNA level in experimental group versus that in the control.

Cytotoxicity and apoptosis assays

Cytotoxicity of cisplatin was determined using MTT and colony formation assays as previously described (Li *et al.*, 2010; Liu *et al.*, 2008; Liu *et al.*, 2006; Xu *et al.*, 2007; Yang *et al.*, 2002; Yang *et al.*, 2007; Yin *et al.*, 2010). Briefly, stable cell lines or cells harvested from transient transfection were cultured in 96-well plates at a density of 2000 cells/well for 24 hours and then treated with cisplatin at various concentrations for three days (MTT) or 8–11 days (colony formation). Viable cells were stained with MTT followed by determination of $OD_{570\ nm}$ with a reference wavelength at 630 nm or stained with crystal violet for colony counting. The data were analyzed using GraphPad Prism4 (GraphPad Software, La Jolla, CA) to obtain an IC_{50}. The relative resistance factor was calculated by dividing the IC_{50} of cells with altered eIF3a expression by the IC_{50} of control cells.

Apoptosis analysis by DAPI or Hoechst 33342 staining of disintegrated nuclei was performed as previously described (Koo *et al.*, 1999). Briefly, both floating and adherent cells following cisplatin treatment were harvested and stained with DAPI or Hoechst dye. The stained cells were mounted onto a polylysine-coated slide by centrifugation and examined under a fluorescent microscope. A total of 300~400 nuclei from five randomly chosen fields were examined and the nuclei displaying distinctive apoptosis-associated morphologic changes were scored. Apoptosis was expressed as a percentage of the total number of nuclei examined.

Apoptosis analysis using Cell Death Detection ELISA kit (Roche, Indianapolis, IN) was performed as instructed by manufacturer. Briefly, S16 cells were plated in six-well plate at 2.5×10^5 cell per well and cultured for 24 hours before being transfected with eIF3a or scrambled control siRNA. Forty-eight hours after transfection, media were replaced with fresh complete media and the cells were irradiated with various doses of UV light followed by continuous culture for 24 hours. Cells were then harvested and about 1×10^4 cells were lysed and analyzed for apoptosis. PARP cleavage, indicator of apoptosis, was analyzed using Western blot probed with an antibody that detects mainly the large fragment of cleaved PARP to avoid potential confusion from detection of uncleaved PARP.

Host-cell reactivation assay (HCR)

The firefly luciferase assay-based HCR was performed as previously described (Qiao *et al.*, 2002). Briefly, the pCMV*luc* plasmid (50 μg/ml) in a 48-well plate was UV irradiated on ice using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA, USA). UV-induced damages were verified using PCR as previously described (Yin *et al.*, 2010). The UV-damaged or un-irradiated control plasmids (0.2 μg) were used to transfect 4×10^4 cells in a 24-well plate using Lipofectamine. A pCMV-SPORT-βgal (Promega, Madison, WI, USA) plasmid encoding β galactosidase was used as a control for transfection efficiency. Forty hours after transfection, cells were harvested for analysis of firefly luciferase and β-galactosidase activities as previously described (Dong *et al.*, 2004).

Pulse and pulse-chase labeling of cells

Pulse and pulse-chase labeling of cells were performed as previously described (Dong *et al.*, 2004; Dong and Zhang, 2003). Briefly, the transiently transfected cells were cultured for additional 48 hrs and washed twice with PBS, once with DMEM medium lacking methionine, followed by incubation for 2 hrs in the same medium supplemented with 7.5 μCi/ml [^{35}S]methionine. The pulse-labeled cells were then washed three times with PBS and harvested for cell lysate preparation and immunoprecipitation. To chase the labeling, cells were washed twice with PBS and once with DMEM medium following the 2 hr pulse-labeling. The cells were then cultured in DMEM medium supplemented with 100 μg/ml cold methionine up to 8 hours. After washing three times with PBS, the cells were harvested for cell lysate preparation and immunoprecipitation.

Immunoprecipitation

Following pulse or pulse-chase labeling, cells were harvested and lysed in TNN-SDS buffer without DTT as described above. The lysates were then incubated with 1 μg of normal mouse IgG at 4° C for 2 hrs followed by incubation with 50 μl of 50% protein G-agarose slurry at 4° C for addition 3 hrs and centrifugation at 500×g for 5 min to remove non-specific bound proteins. The supernatants were then incubated with 5 μg of primary antibodies against XPA, XPC, RAD23B, and RPA32 at 4° C for 2 hrs before mixing with 50 μl of protein G-agarose beads. The mixtures were incubated at 4° C overnight followed by washing for five times with lysis buffer. The final precipitates were separated by SDS-PAGE and signals were detected by ECL system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The cisplatin-sensitive clone S16 was established and characterized at Van Andel Research Institute. The stable CNE-2 cells with eIF3a over-expression and SUNE-1 cell line were studied at Sun Yet-sen University Cancer Center. All other studies were performed at Indiana University School of Medicine. The VARI team would like to dedicate this paper to the memory of a friend and colleague, Dr. Han-Mo Koo. This work was supported in part by the National Institutes of Health grant CA94961 (JTZ), by Showalter Trust Fund (ZD), and by National Natural Science Foundation of China (No. 81071822) (RYL).

Abbreviations used

CDDP	cisplatin
eIF	eukaryotic initiation factor
NER	nucleotide excision repair
NPC	nasopharyngeal carcinoma
RPA	replication protein A
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C

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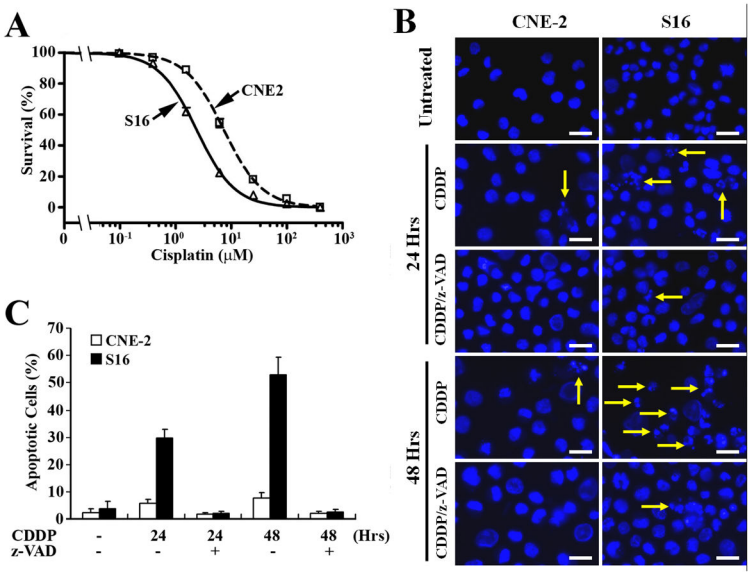

Figure 1. Characterization of the cisplatin sensitive CNE2 derivative subline S16 cells

A, survival assay. S16 and its parental CNE2 cells were treated with various concentrations of cisplatin (CDDP) for 72 hrs followed by MTT assay for cell survival. B and C, apoptosis assay. S16 and its parental CNE-2 cells were pretreated with 30μM pan-caspase inhibitor z-VAD-fmk 2 hrs prior to treatment with cisplatin (CDDP) at 8.7 μM for 24 or 48 hrs followed by DAPI staining of nuclei and imaging analysis as described in Materials and Methods. Yellow arrows indicate apoptotic cells. Scale bar=25 μm. Panel C shows statistical analysis of apoptotic cells generated from three independent experiments.

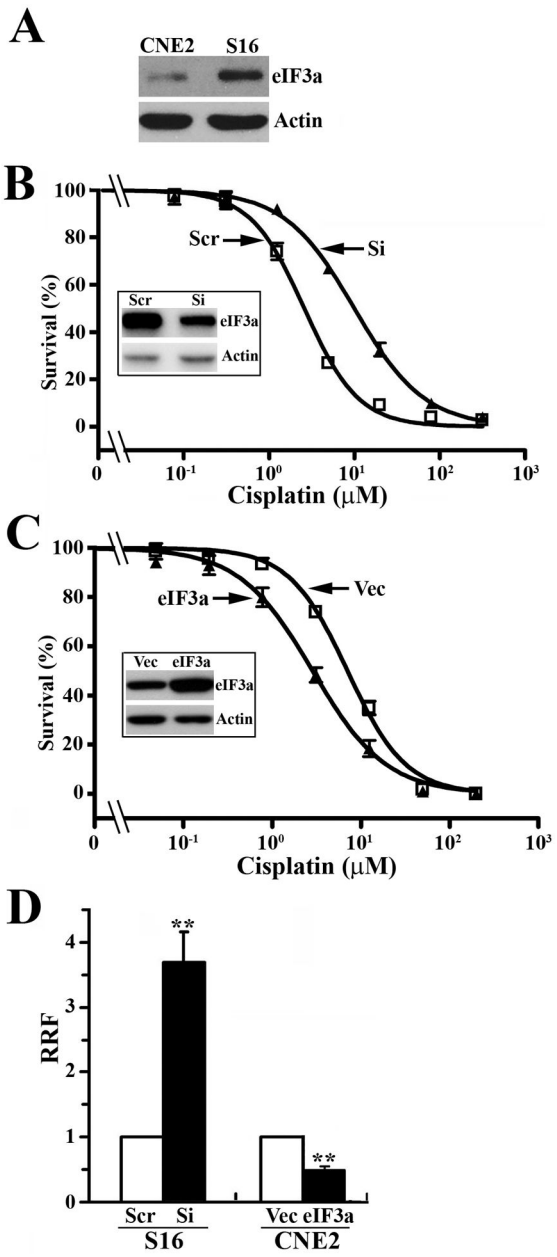

Figure 2. Contribution of eIF3a over-expression to cisplatin sensitivity of S16 cells

A, eIF3a expression in S16 and CNE-2 cells. CNE-2 and S16 cells in logarithmic growth phase were collected and lysed followed by Western blot analysis of eIF3a and actin loading control. B and C, effect of eIF3a knockdown or ectopic over-expression on cisplatin sensitivity. S16 cells (B) transiently transfected with eIF3a siRNA (Si) or scrambled control siRNA (Scr) or CNE2 cells (C) with stable eIF3a over-expression (eIF3a) or transfected with vector control (Vec) were analyzed for their eIF3a expression level (inset) and cisplatin response as described in Materials and Methods. D, relative resistance factor. Relative resistance factor (RRF) was derived by dividing the IC_{50} of the cells with eIF3a knockdown

(for S16 cells) or over-expression (for CNE2 cells) by that of the control cells generated from 3–5 independent experiments (**=p<0.01).

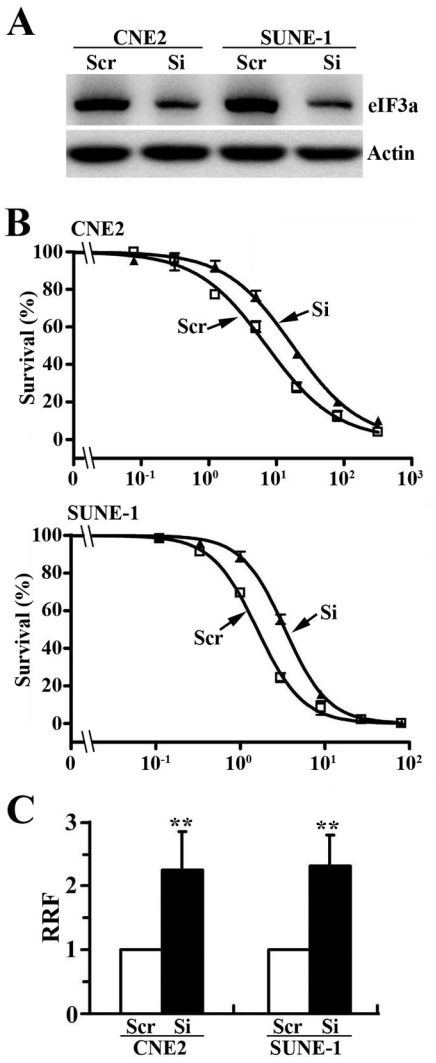

Figure 3. Role of eIF3a in cisplatin response of NPC cell lines SUNE-1 and CNE2

SUNE-1 and CNE2 cells were transiently transfected with eIF3a siRNA (Si) or scrambled control siRNA (Scr) followed by Western blot analysis of eIF3a expression (A) and MTT assay of cellular response to cisplatin (B). Panel C shows relative resistance factor (RRF) derived by dividing IC_{50} of the cells with eIF3a knockdown by that of the control cells from 3–5 independent experiments (**=$p<0.01$).

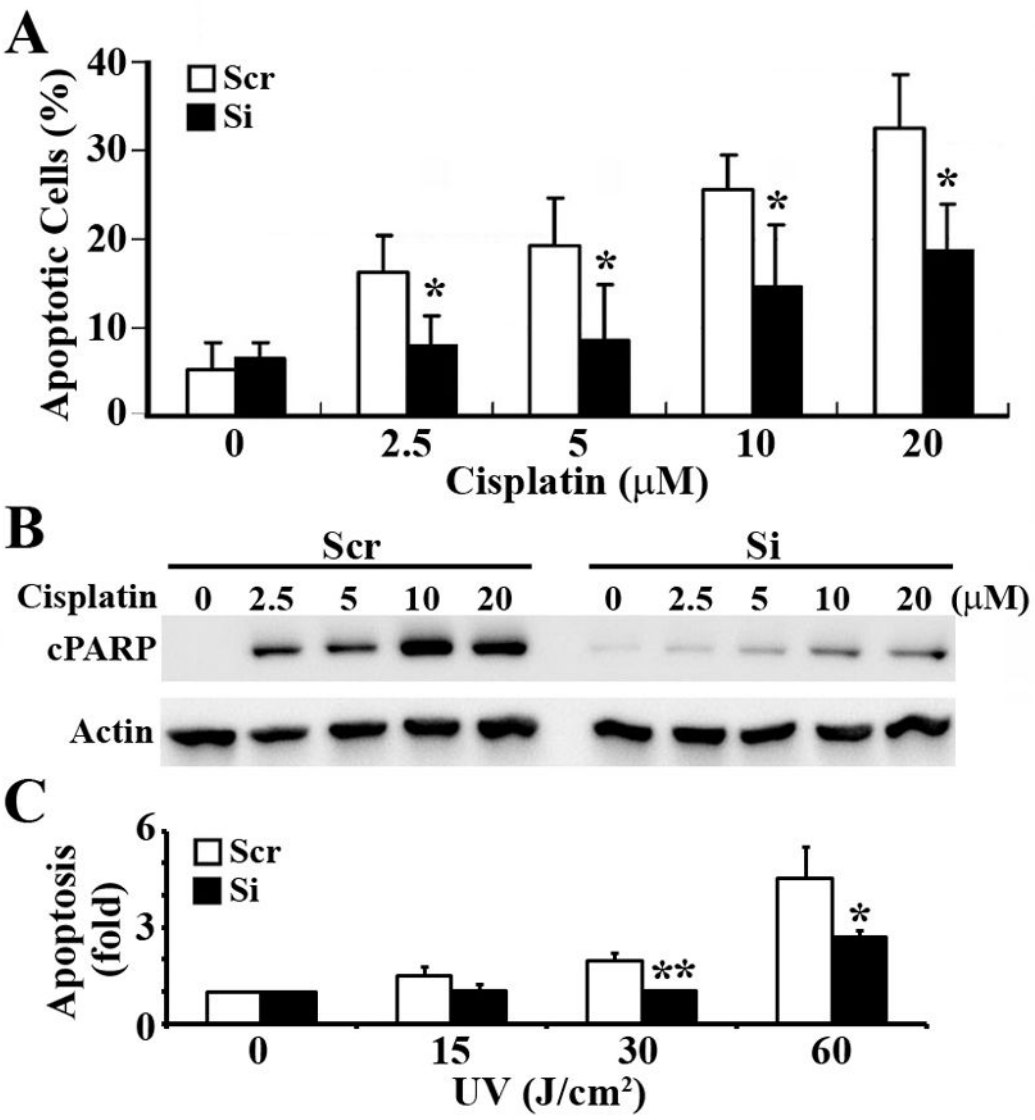

Figure 4. Effect of eIF3a knockdown on DNA-damage-induced apoptosis

S16 cells were transiently transfected with eIF3a siRNA (Si) or scrambled control siRNA (Scr) followed by treatment with cisplatin (A & B) or UV (C) at doses indicated and analysis of apoptosis using Hoechst 33342 staining for disintegrated nuclei (A), detection of cleaved PARP on Western blot (B), or Cell Death Detection ELISA kit (C) as described in Materials and Methods. The histograms shown are from three independent experiments (*=$p<0.05$; **=$p<0.01$).

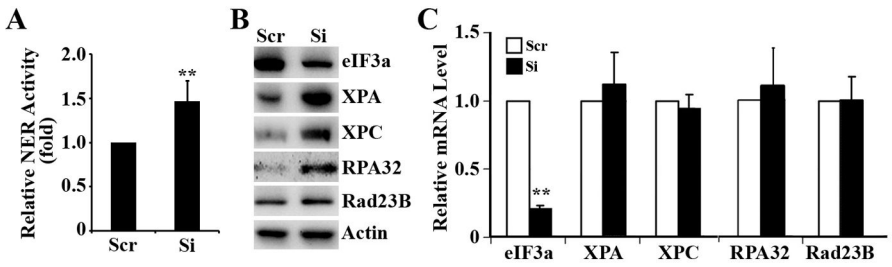

Figure 5. Effect of eIF3a knockdown on NER activity and expression of NER proteins

S16 cells were transiently transfected with eIF3a siRNA (Si) or scrambled control siRNA (Scr) followed by analysis of NER activity using host cell reactivation assay (A), expression of eIF3a, XPA, XPC, RPA32, and RAD23B using Western blot (B) and real time RT-PCR (C) analyses as described in Materials and Methods. Actin was used as a loading control for Western blot and GAPDH was used as an internal standard for real time RT-PCR analyses.

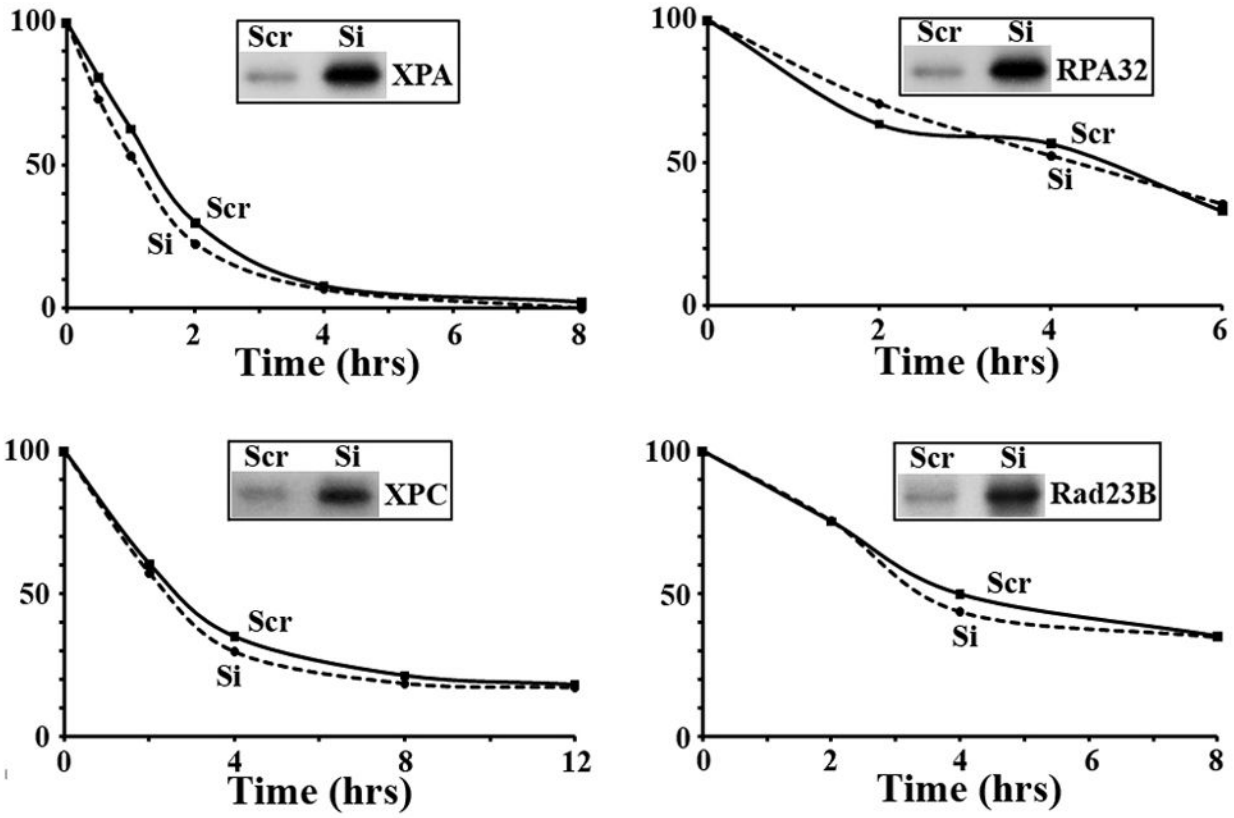

Figure 6. Effect of eIF3a knockdown on synthesis and half-life of NER proteins

S16 cells were transiently transfected with eIF3a siRNA (Si) or scrambled control siRNA (Scr) followed by pulse labeling with [^{35}S]methionine and preparation of cell lysate or washed and chased in the presence of cold methionine for various times before preparation of cell lysate as described in Materials and Methods. The cell lysates were then subjected to immunoprecipitation of XPA, XPC, RPA32, and RAD23B followed by separation on SDS-PAGE and autoradiaography of the gels. The insets show the results following pulse labeling (synthesis) and the curves show the results of chase (half-life).