The Inhibition of Endoplasmic Reticulum Chaperone Protein GRP78 Enhances the Anti-tumor Effect of Sunitinib in Renal Cell Carcinoma Model

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Directed by Professor Sung Joon Hong

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Dong Suk Kim

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ABSTRACT

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Tumor microenvironments are characterized by less oxygen and nutrition than normal tissues due to the rapid and progressive nature of the tumors. These intense cell stressors trigger a protective cell survival mechanism heralded by the unfolded protein response (UPR). The UPR is induced by an accumulation of unfolded proteins in the endoplasmic reticulum (ER) after cell starvation and leads to the global suppression of mRNA translation to protect cells from excessive amounts of unfolded proteins. Although the ER stress response is implicated in cytoprotection, its precise role remains unclear in renal cell carcinoma. One of the major proteins involved in ER stress is glucose-regulated protein 78 (GRP78), which binds to unfolded proteins and dissociates from membrane-bound ER stress sensors. To determine the role of ER stress responses during anti-angiogenic therapy and the potential role of GRP78 for use in single or combined therapy in renal cell carcinoma (RCC), we used transient and stable GRP78-knockdown RCC cells under hypoxic or hypoglycemic stress conditions in vitro and in animal models treated with sunitinib. Here, we report that ER protein GRP78 plays a crucial role in protecting RCC cells from hypoxic and hypoglycemic stress in tumor microenvironments. GRP78 overexpression led to increased tumor proliferation following hypoxic and/or hypoglycemic stress by activating the double-stranded RNA-activated protein kinase-like ER kinase/eukaryotic initiation factor-2α pathway and protected tumor cells from stress-induced

apoptosis. Knockdown of GRP78 using small interfering RNA (siRNA) inhibited cancer cell survival and induced apoptosis in RCC cells in vitro. GRP78 knockdown showed more potent suppression of tumor progression and enhanced the anti-tumor effect on sunitinib in vitro and in vivo. Our findings suggest that knockdown of GRP78 enhances apoptotic cell death from anti-angiogenic stress and that GRP78 may be a novel therapeutic target in RCC management.

Key words : GRP78, endoplasmic reticulum stress, renal cell carcinoma, sunitinib

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I. INTRODUCTION

Recently developed targeted therapies have shown promising results in the treatment of advanced malignancies. In particular, anti-angiogenic therapies that target vascular endothelial growth factor (VEGF) signaling have demonstrated remarkable clinical benefits in patients with advanced renal cell carcinoma (RCC)[1-3]. Targeting tumor angiogenesis induces severe tumor starvation, and then oxygen and nutrition deprivation successfully inhibits tumor growth and leads to disease stabilization. Sunitinib, one of the most efficacious multi-tyrosine kinase inhibitors targeting VEGF, significantly prolongs progression-free survival in patients with advanced RCC and is the first-line therapy for metastatic RCC[1]. However, anti-angiogenic therapy is generally not cytotoxic but rather cytostatic; thus, tumors shrink and regress, but many tumor cells survive anti-angiogenic treatment and resume growth after an interval of stability. Once the tumor acquires resistance to the anti-angiogenic agent, the tumor rapidly progresses and the prognosis is very poor even with second and third line treatment options.

Tumors are often exposed to harsh microenvironments. Due to their rapid and progressive nature, tumors are surrounded by limited resources such as oxygen

and nutrients. Moreover, many cancer therapeutics deprive tumors of essential resources for cancer cell survival. These intrinsic and extrinsic environmental stressors generally induce the accumulation of unfolded proteins in the endoplasmic reticulum (ER) of cancer cell[4-5]. There, they elicit the unfolded protein response (UPR), which is a general cellular defense mechanism that dampens non-essential global protein synthesis to protect cells from stress. Many ER-resident proteins display an altered expression pattern in cancer.

One of the most abundant ER-resident proteins is the ER chaperone glucose-regulated protein 78 (GRP78), also known as immunoglobulin heavy-chain-binding protein (BiP)[6]. A major UPR response is the induction of GRP78/BiP, which is highly expressed in a variety of tumors, including prostate cancer, melanoma, and head and neck cancer[7], and confers drug resistance in both proliferating and dormant cancer cells. GRP78 binds to unfolded proteins and dissociates from the membrane-bound ER stress sensors; dissociation of GRP78 allows the subsequent activation of the stress sensors, which suppresses global mRNA translation to protect cells from excessive amounts of unfolded proteins[8]. This adaptive response activated on accumulation of unfolded proteins in the ER is mediated by at least three ER proximal sensors, PKR-related ER kinase (PERK), inositol requiring enzyme-1 (IRE1), and activating transcription factor (ATF) 6[9]. PERK is a serine threonine kinase that phosphorylates eukaryotic translation initiation factor 2A (eIF2a), a translation initiation factor that functions in the early steps of protein synthesis by forming a ternary complex with GTP and initiator tRNA, on stress and consequently attenuates global protein translation[9]. A recent study revealed that GRP78 is highly expressed in RCC cells and suggested a significant relationship between GRP78 expression and clinicopathological features of RCC, including tumor size, histological grade, and stage[10]. In addition, anti-vascular and anti-angiogenic therapies reportedly result in severe glucose and oxygen deprivation, which could lead to GRP78 induction in residual tumor cells and result in drug resistance[11].

GRP78 protects tumor cells from apoptosis under several types of stress

conditions, including hypoxia, nutrient deprivation, and cytotoxic chemotherapy [12-14]. As well, expression of GRP78 has also been shown to be a predictor of poor outcomes in RCC. However, a precise role of GRP78 in renal cell carcinoma following anti-angiogenic therapy remains unclear. Here, we show that ER stress responses are activated in tumor cells and highly activated during anti-angiogenic therapy and that the inhibition of the UPR by knockdown of GRP78 suppresses tumor progression and enhances the anti-tumor effects of anti-angiogenic therapy in RCC in vitro and in vivo. Our results suggest that GRP78 may be a novel therapeutic target in the current therapeutic strategy for RCC.

II. MATERIALS AND METHODS

1. Cell Culture and Reagents

Two human RCC cell lines, Caki-1 and Caki-2, were obtained from the American Type Culture Collection (ATCC) and were maintained in McCoy's 5A Medium (HyClone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. Two other human RCC cell lines, UMRC-3 and UMRC-6, were kindly provided by Dr. P. Black (Vancouver Prostate Centre, University of British Columbia, Vancouver, Canada) and were maintained in Minimal Essential Medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. All cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2. For all studies, cell lines were passaged for a maximum of 2 months.

2. Transient Transfections

To develop cells with transient GRP78 knockdown, Caki-1 and UMRC-3 cells were transiently transfected with a 19-bp small interfering RNA (siRNA) targeting GRP78 (sense: 5′-GGAGCGCAUUGAUACUAGA-3′, anti-sense: 5′-GCCUAGGUCUCUUAGAUGA-3′) or a non-silencing siRNA (si-Scr)[14]. Caki-1 and UMRC-3 cells were seeded on six-well plates at a density of 1.2×10^5 cells/well, and cells at 30-50% confluence were transfected for 16 h with the siRNAs using Lipofectamine 2000 (Invitrogen Life Technologies) diluted with OPTIMEM (Invitrogen Life Technologies) according to the manufacturer's instructions. After transfection, the medium was replaced with fresh medium, and the cells were incubated for 48-72 h according to the purpose of the experiment. To develop cells with transient protein kinase-like ER kinase (PERK) knockdown, GRP78-overexpressing Caki-1 and parental Caki-1 cells were transfected with PERK siRNA (Santa Cruz Biotechnology).

3. Cloning and Stable Transfections

To generate GRP78-overexpressing Caki-1 cells, the pHR-CMV lentivirus

system was used as described previously[15]. Briefly, human GRP78 cDNA was subcloned into pHR-CMV lentiviral vector. 293T cells were then transiently transfected with GRP78-pHR-CMV, along with packaging vectors, VSVG and R8.91. 48 hours after transfection, lentivirus containing culture media was collected and applied to Caki cells for transduction. To develop Caki-1 GRP78 knockdown cells stably expressing shGRP78, the pLKO.1 lentivirus system was used. The oligonucleotides for GRP78 small hairpin RNA (shRNA) were synthesized and cloned into pLKO.1 by Sigma Chemical Company. ShGRP78-overexpressing Caki-1 clones were selected with puromycin (Invitrogen). To generate stable tetracycline-inducible knockdown of GRP78 in Caki-1 cells, pSingle-tTS-shRNA vector system (Clontech) was used. GRP78-specific and control non-targeting shRNA sequences were designed following the manufacturer's protocol: for GRP78 shRNA,sense, 5′-TCGAGGGGAGCGCATTGATACTAGATTCAAGAGATCTAGTATCAA TGCGCTCCTTTTTTACGCGTA-3′ and antisense, 5′-AGCTTACGCGTAAAAAAGGAGCGCATTGATACTAGATCTCTTGAA TCTAGTATCAATGCGCTCCCC-3′;for non-targeting shRNA, sense, 5'-TCGAGG AGTTCAACGAGTATCAGCATTCAAGAGATGCTGATACTCGTTGAACT TTTTTTACGCGTA-3' and antisense,. 5'-AGCTTACGCGTAAAAAAAGTTCAACGAGTATCAGCATCTCTTGAA TGCTGATACTCGTTGAACTCC-3'Annealed double-stranded shRNA oligonucleotides were cloned into XhoI/HindIII site of pSingle-tTS-shRNA vector. Cloning was confirmed by nucleotide sequencing. The pSingle-tTS GRP78 shRNA or pSingle-tTS non-silencing control shRNA was transfected into Caki-1 cells using Xtreme Gene 9 (Roche) diluted with OPTIMEM according to the manufacturer's instructions. Transfected cells were selected using 400 μg/mL G418 (Roche) and were maintained in medium containing 200 μg/mL G418. Stable cell clones were then confirmed for doxycycline-induced GRP78 gene silencing by both Western blot analysis and reverse

transcription-polymerase chain reaction (RT-PCR). After polyclonal selection and confirmation, stable cell lines were frozen at early (less than 10) passages.

4. Cell Viability Assay

Cells were seeded on six-well plates at 1.2×10^5 cells per well, incubated overnight, and transfected. After incubation for another 72 h, the cells were fixed with 1% glutaraldehyde and stained with a 0.5% crystal violet solution. The cells were washed with water, and any remaining crystal violet was resolved with Sorensen's solution. Absorbance was measured at 562 nm on a spectrophotometer. All experiments were performed in triplicate.

5. Cell Cycle Analysis and Apoptosis

Cells were harvested and fixed with 70% alcohol as a single-cell suspension. The fixed cells were collected by centrifugation and washed with phosphate-buffered saline (PBS). The cells were incubated with RNase A (1 mg/mL) for 30 min at 37℃ and then incubated with propidium iodide (20 μg/mL; Sigma Chemical) for 30 min. Relative DNA contents were analyzed by FACSCanto II flow cytometer and FACSDiva software (BD Bioscience).

6. Western Blot Analysis

Cellular proteins were harvested in RIPA lysis buffer containing vanadate, phosphatase inhibitor, and phenylmethanesulfonyl fluoride. The proteins were resolved by electrophoresis in polyacrylamide gels and transferred to a nitrocellulose membrane using a Trans-Blot system (BioRad) as previously described[14]. The membranes were blocked in 5% skim milk diluted with Tris-buffered saline and Tween 20 (TBST) for 1 h at room temperature, and were then incubated with primary antibodies diluted in 3% skim milk in TBST for 1 h at room temperature or overnight at 4℃. Antibodies against the following proteins were used for Western blotting (all from Santa Cruz Biotechnology): GRP78 (C-20) (diluted 1:1000), PERK (1:1000), p-PERK (1:250-500), p-eIF2a (1:250-500), GADD153 (1:500), CREB-2 (1:500), and

vinculin (1:5000). Anti-goat, anti-rabbit, and anti-mouse IgG secondary antibodies (1:5000) were used. Amersham enhanced chemiluminescence reagents (GE Healthcare) or Supersignal chemiluminescence reagents (Thermo Scientific) were used to detect immunoreactive proteins on the Western blots.

7. Immunofluorescence

Cells were seeded on glass coverslips in six-well plates and allowed to attach overnight. To evaluate baseline expression of GRP78 in wild-type RCC cells and GRP78-overexpressing cells, the cells were washed with ice-cold PBS, fixed with ice-cold methanol mixed with acetone (3:1) for 10 min at room temperature, and treated with 0.25% Triton X-100 in PBS for 10 min. After blocking with 3% bovine serum albumin for 30 min, the cells were incubated with anti-GRP78 (C-20) antibody at room temperature for 1 h, washed three times with Tris-buffered saline, and incubated with Alexa Fluor 488-conjugated anti-goat antibody for 1 h in a dark room. Nuclei were stained with DAPI. Immunofluorescence was imaged with a Zeiss confocal laser scanning microscope.

8. In Vivo Xenograft Models

All animal experimentation was conducted in compliance with accepted standards of the University of British Columbia Committee on Animal Care. General anesthesia of mice was induced and maintained by inhaled isoflurane. Six- to eight-week-old female nu/nu nude mice were subcutaneously injected in the flank area with a cell suspension containing 3×10^6 tumor cells. Caki-1 cells were used to evaluate GRP78 expression after sunitinib treatment. Caki-1 cells stably expressing doxycycline-inducible shGRP78 were used to investigate the effect of in vivo knockdown of GRP78. When the tumor size reached 150-250 mm^3, the mice were randomized to receive vehicle, sunitinib, doxycycline, or sunitinib and doxycycline for 30 days (n = 8 mice per group). Sunitinib was administered by oral gavage as a citrated-buffered (pH 3.5) solution once daily, at the dosage of 40 mg/kg. Every 3 days, tumor size was measured using calipers

and body weight was determined. Tumor volume was calculated as length × width × height × 0.5. After treatment was complete, the mice were euthanized in a CO_2 chamber, followed by cervical dislocation. The tumors were fixed in 10% formalin for immunohistochemical staining or frozen at −80°C for protein and RNA analyses.

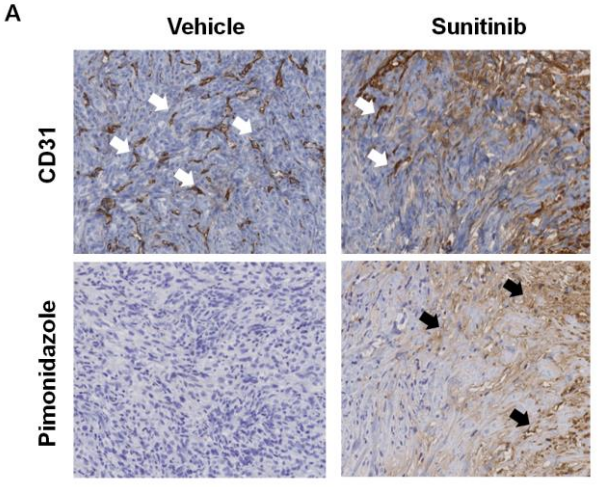
9. Immunohistochemistry

Formalin-fixed, paraffin-embedded tumors were cut into 4-μm sections, which were deparaffinized, rehydrated with xylene and ethanol, and transferred into 0.02% Triton for permeabilization. Slides in citrate buffer (pH 6) were heated in a steamer for 3 min, washed in PBS for 5 min, incubated in 3% bovine serum albumin, transferred into 3% H_2O_2, and incubated with anti-GRP78 antibody overnight.

III. RESULTS

1. GRP78 expression is upregulated by hypoxic and hypoglycemic stress in RCC cells

To determine whether GRP78 is upregulated during anti-angiogenic therapy, we treated Caki-1 xenografts with sunitinib, a multi-tyrosine kinase inhibitor and potent anti-angiogenic agent, and then used immunohistochemical staining to examine the change in GRP78 expression in xenografts. Figure 1A shows that sunitinib treatment induced intratumoral hypoxia in the xenografts by decreasing intratumoral microvessels. After 5-day sunitinib treatment, GRP78 expression was higher than the level in the control group. GRP78 was also upregulated in the control group during a later stage; however, the treatment group still displayed higher GRP78 expression, suggesting that ER stress may be induced by oxygen or nutrient deprivation (Fig. 1B). To further determine whether ER stress induced by anti-angiogenic therapy is due to hypoxic stress, hypoglycemic stress, or a combination of both stressors, we examined GRP78 expression in Caki-1 cells after exposure to hypoxic and/or hypoglycemic conditions. Western blot and immunofluorescence analyses indicated that GRP78 was upregulated by hypoxia, hypoglycemia, and both (Fig. 1C and D).



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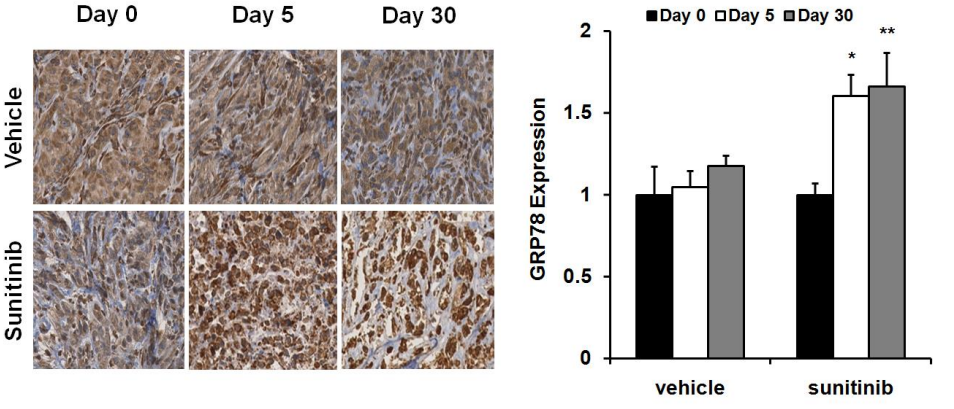
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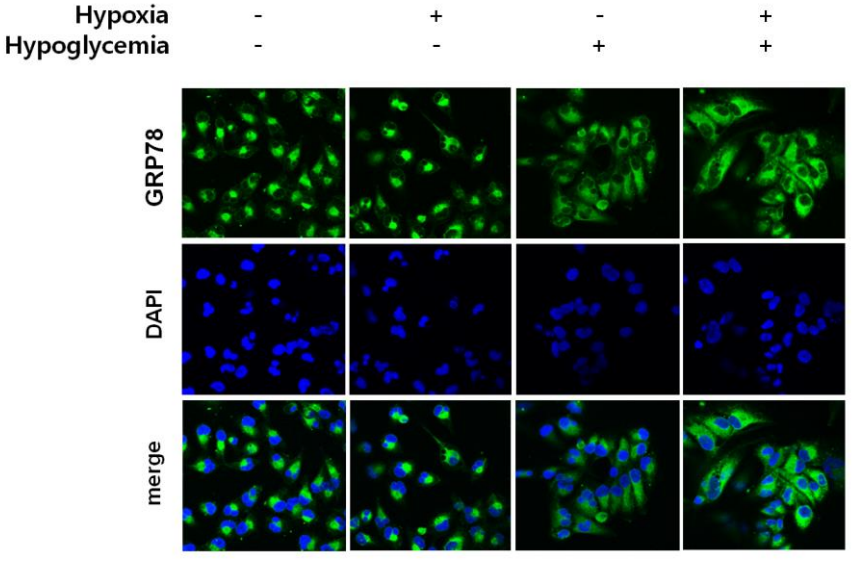
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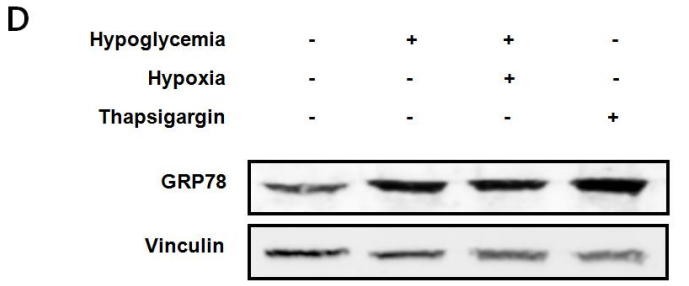


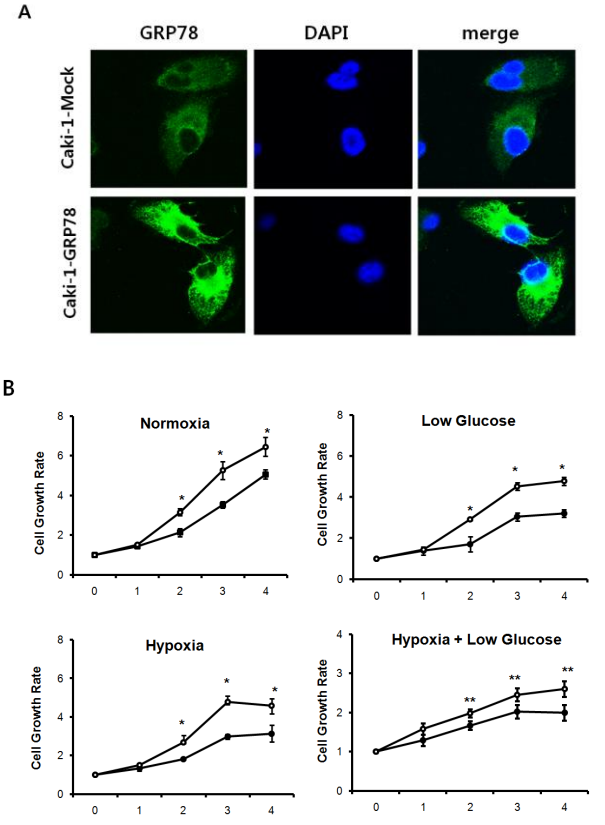
Figure 1. Expression of GRP78 protein during hypoxia and glucose deprivation in renal cell carcinoma cells.

Sunitinib treatment induces hypoxia by suppressing microvessels in Caki-1 xenografts, white arrows indicate microvessels and black ones indicate hypoxic area (A). GRP78 is overexpressed in xenografts after treatment with sunitinib as compared with vehicle treatment *, P = 0.003 vs. vehicle (day 5). **, P = 0.004 vs. vehicle (day 30). (B). GRP78 is highly expressed in the endoplasmic reticulum (ER) of renal cell carcinoma cells during hypoxia and/or glucose deprivation (C and D).

2. GRP78 upregulation promotes cancer cell proliferation by activating the PERK/eIF2α pathway in RCC cells

To confirm the role of GRP78 in tumor cell survival and proliferation in RCC, we transfected Caki-1 cells with GRP78-encoded lentivirus (Caki-1-GRP78) or empty vector lentivirus (Caki-1-Mock). Immunofluorescence imaging showed that GRP78 was stably expressed at a higher level in Caki-1-GRP78 cells than in Caki-1-Mock cells (Fig. 2A). GRP78 overexpression promoted cell proliferation in Caki-1 cells, as demonstrated by the crystal violet cell viability assay (Fig. 2B). We next performed a cell growth assay under hypoxic and/or hypoglycemic conditions, representing intratumoral stress conditions induced by anti-angiogenic therapy. Cell proliferation was enhanced in GRP78-overexpressing cells during hypoxia or hypoglycemia (Fig. 2B). Western blot analysis of proteins downstream of GRP78 in

Caki-1-GRP78 cells revealed that GRP78 upregulation activated PERK through phosphorylation, increased CREB-2, and decreased C/EBP homology protein (CHOP) (Fig. 2C). To further determine whether GRP78 promotes tumor proliferation via PERK pathway activation, we knocked down PERK in Caki-1-GRP78 cells using PERK siRNA and examined cell viability. The enhanced proliferation returned to a normal level, as in Caki-1-Mock cells (Fig. 2D). These results indicate that GRP78 promotes cancer cell survival by activating the PERK/eIF2α pathway in RCC cells.



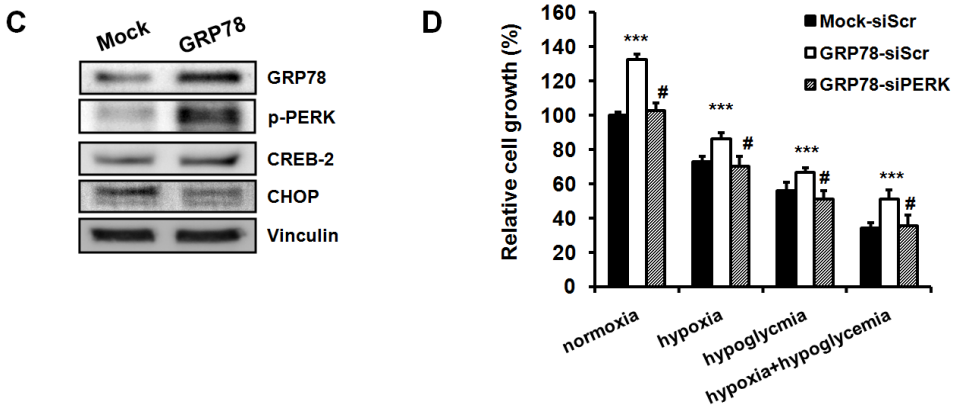


Figure 2. Effect of GRP78 overexpression on tumor growth, cell cycle, and downstream effectors in renal cell carcinoma cells.

Caki-1 cells were stably transfected using pHR-CMV-GRP78 or –mock (A) Expression of GRP78 is significantly higher in the Caki-1-GRP78 cells relative to Caki-1-Mock cells. Overexpression of GRP78 increases tumor growth in normoxia, hypoxia, and/or hypoglycemia, * P <0.001 vs. mock, ** P <0.01 (B) GRP78 overexpression induces the phosphorylation of PERK and CREB-2 but reduces GADD153 in Caki-1 cells (C). Increased proliferation shown in Caki-1-GRP78 cells returned to a normal level (Caki-1-mock cells) after knockdown of downstream effetor, PERK using siRNA. *** P<0.001 vs. Mock-siScr, # P>0.05 vs. Mock-siScr (D)

3. GRP78 protects RCC cells from stress-induced apoptosis

We next studied the role of GRP78 upregulation in apoptotic cell death in RCC cells by inducing apoptosis with staurosporine. Caki-1-GRP78 or Caki-1-Mock cells were treated with 0.3 μM staurosporine for 48 h, and then cell cycle analysis was performed with flow cytometry. GRP78 did not reduce apoptosis following dimethyl sulfoxide (DMSO) treatment, but significantly reduced the percentage of apoptotic nuclei after staurosporine exposure (Fig. 3A and B). To confirm the anti-apoptotic effect of GRP78 in RCC cells, we examined the activation of caspase-3 and poly (ADP-ribose) polymerase (PARP) using Western blot analysis. Caspase-3 and PARP were not induced by DMSO

treatment, whereas they were markedly induced following staurosporine treatment. GRP78 overexpression reduced the activation of caspase-3 and PARP in Caki-1 cells (Fig. 3C). These data suggest that GRP78 has an anti-apoptotic role in RCC cells.

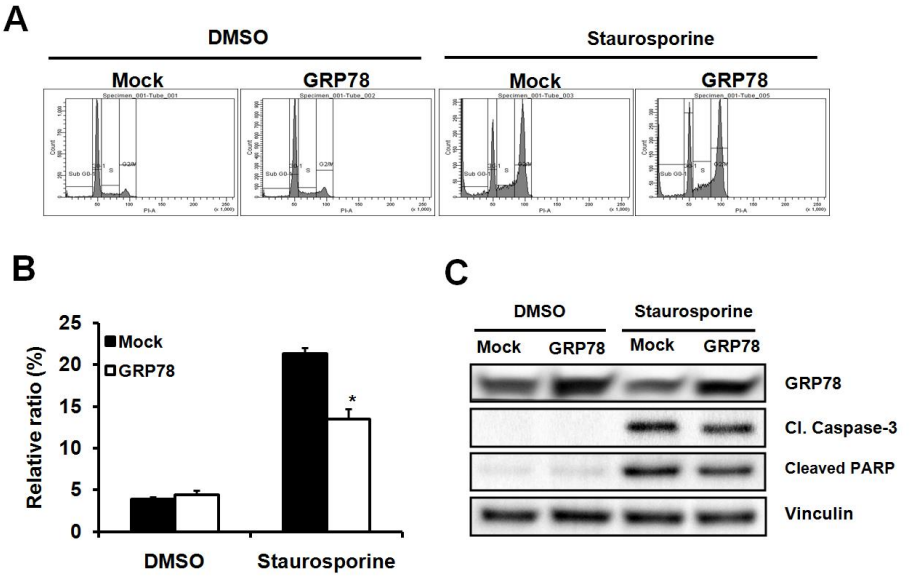
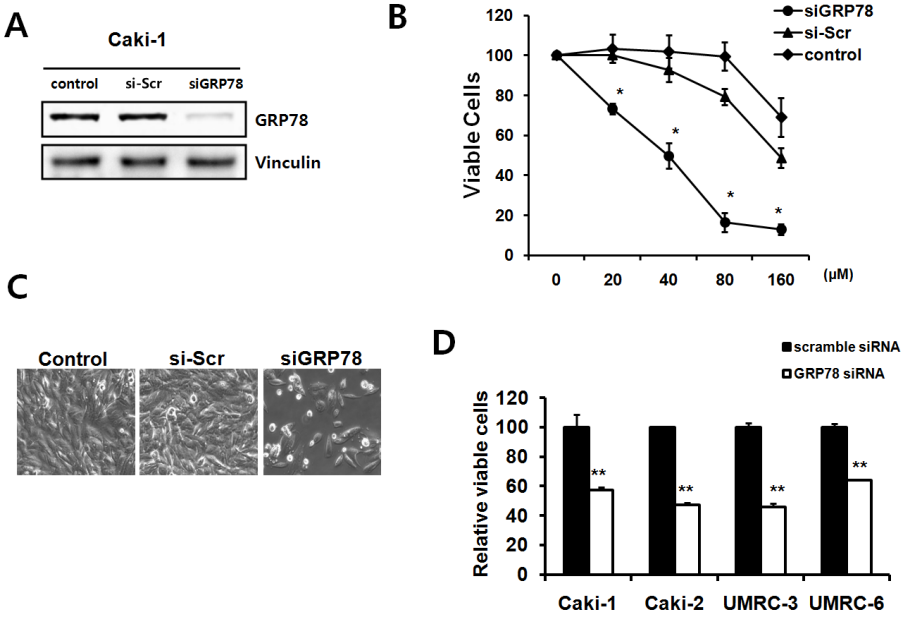


Figure 3. Changes in cell cycle distribution and apoptosis after GRP78 overexpression in renal cell carcinoma cells. GRP78-overexpressing Caki-1 cells were treated with staurosporine to induce apoptosis, stained with propidium iodide (PI), and analyzed by flow cytometry. GRP78 overexpression reduces staurosporine-induced apoptosis in Caki-1 cells, * P <0.01 vs. Mock (A and B). Western blots show reduced levels of cleaved caspase-3 and cleaved PARP (C).

4. GRP78 knockdown induces apoptosis in RCC cells

To study the inhibitory effect of GRP78 on the proliferation of RCC cells, we used GRP78 siRNA to transiently knock down GRP78 expression by >70% in all RCC cell lines (Fig. 4A). GRP78 knockdown inhibited tumor proliferation in all of the RCC cell lines (Fig. 4B-D). To evaluate the effect of GRP78 knockdown on the cell cycle, we examined the cell cycle distribution by flow

cytometry of propidium iodide-stained Caki-1 and UMRC-3 cells. Western blots confirmed that knockdown of GRP78 downregulated the phosphorylation of PERK and eIF2a and the expression of CREB-2, while it induced CHOP expression (Fig. 4E). GRP78 knockdown significantly induced apoptosis in Caki-1 cells (Fig. 4F and G). Western blot analysis showed that caspase-3 and PARP were activated by GRP78 knockdown (Fig. 4F). To determine whether GRP78 knockdown enhances ER stress-induced apoptosis, we used MG132, a proteosome inhibitor inducing apoptosis via ER stress-mediated apoptotic pathway to induce ER stress in Caki-1 cells. MG132 inhibited cell growth and induced apoptosis in Caki-1 cells (Fig. 4G). GRP78 knockdown enhanced MG132-inhibited cell growth and MG132-induced apoptosis. These data demonstrate that GRP78 knockdown suppresses cancer cell survival by inducing apoptosis in RCC cells via the downregulation of PERK and eIF2a.



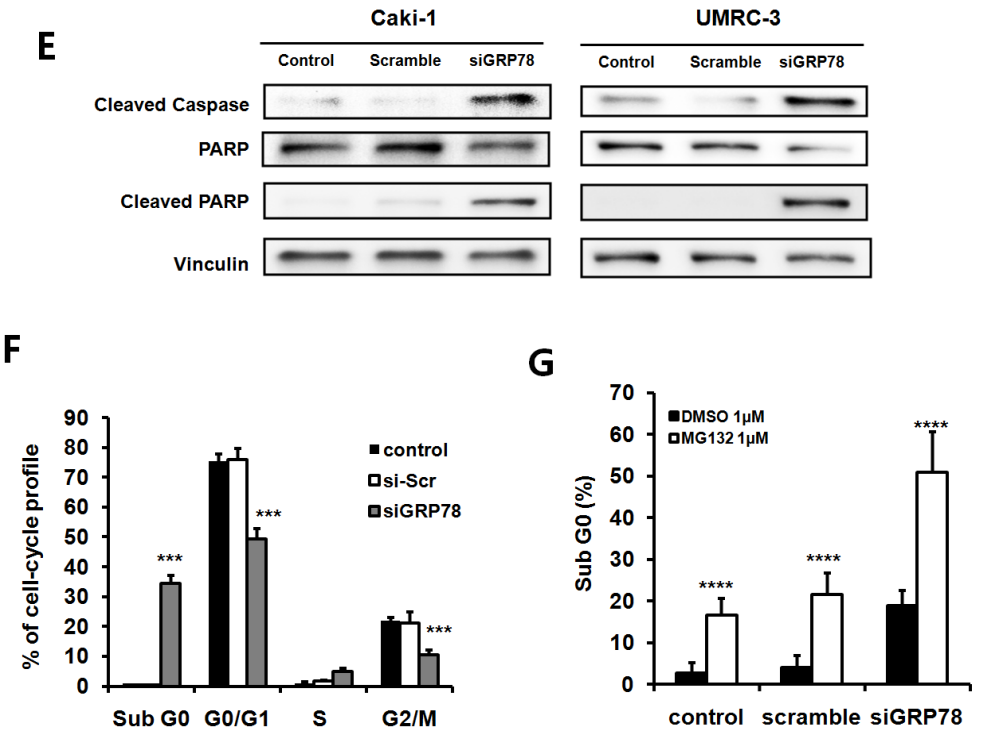
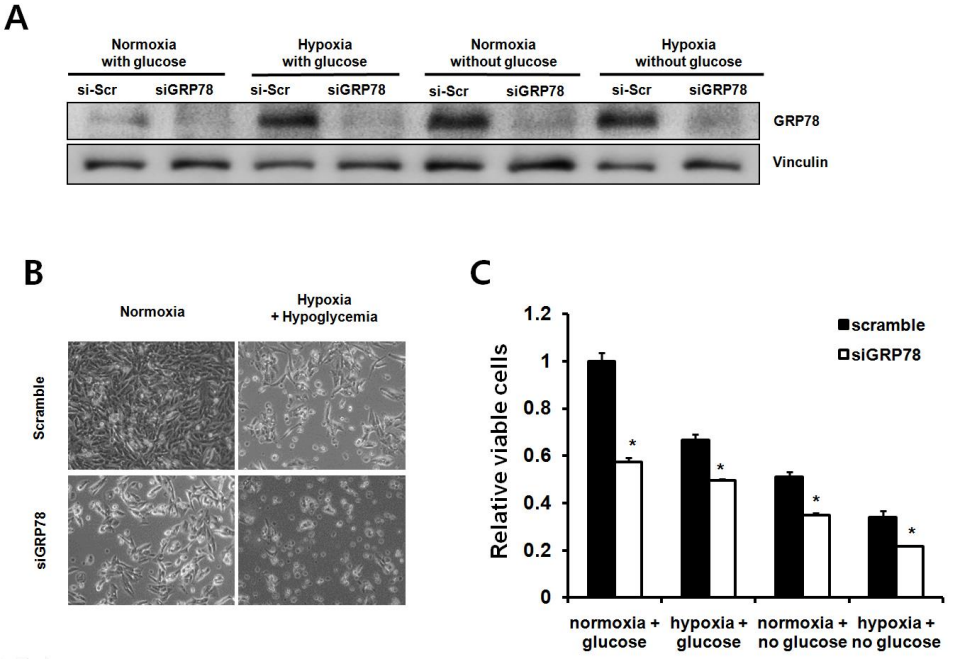


Figure 4. Effects of GRP78 knockdown on tumor growth and downstream effectors of ER stress pathways in renal cell carcinoma cells.

RNA interference using siGRP78 effectively knocks down GRP78 in Caki-1 cells (A). GRP78 knockdown using siRNA inhibits tumor growth in Caki-1 cells, * P <0.001 (B, C). Growth inhibition in vitro is observed in a wide panel of RCC cells, ** P <0.001 vs. scramble siRNA (D). Knockdown of GRP78 using siRNA also induces apoptosis in Caki-1 and UMRC-3 renal cancer cells in vitro. After siGRP78 transfection, Caki-1 and UMRC-3 cells showed increased cleaved caspase and cleaved PARP compared to cells treated with scrambled control siRNA (E). Increased apoptosis was confirmed using flow-cytometry to assess proportion of cells in sub G0 portion of the cell cycle, which was increased in cells treated with siGRP78 compared to siSCR, *** P <0.001 vs. si-Scr (F). Apoptosis was further increased, as shown in the proportion of cells in the sub G0 portion of the cell cycle, combined treatment of MG132 treatment and siGRP78 in Caki-1 cells, **** P < 0.001 vs. DMSO (G).

5. GRP78 knockdown enhance apoptotic cell death by hypoxic and hypoglycemic stress in RCC

We evaluated the effect of GRP78 knockdown on the survival of RCC cells under hypoxic and hypoglycemic conditions. Cell growth was inhibited by hypoxia and hypoglycemia. GRP78 siRNA successfully knocked down GRP78 expression induced by hypoxia and hypoglycemia (Fig. 6A). GRP78 knockdown induced further growth inhibition in addition to that due to hypoxia, hypoglycemia, or both (Fig. 5B and C). To further study the effect of GRP78 inhibition combined with hypoxia or hypoglycemia on cell survival, we examined the cell cycle distribution using flow cytometry. Cell cycle analysis showed that GRP78 knockdown significantly increased the number of apoptotic cells in all conditions (Fig. 5D and E). These data show that the inhibition of GRP78 sensitizes RCC cells to stress from oxygen and glucose deprivation.



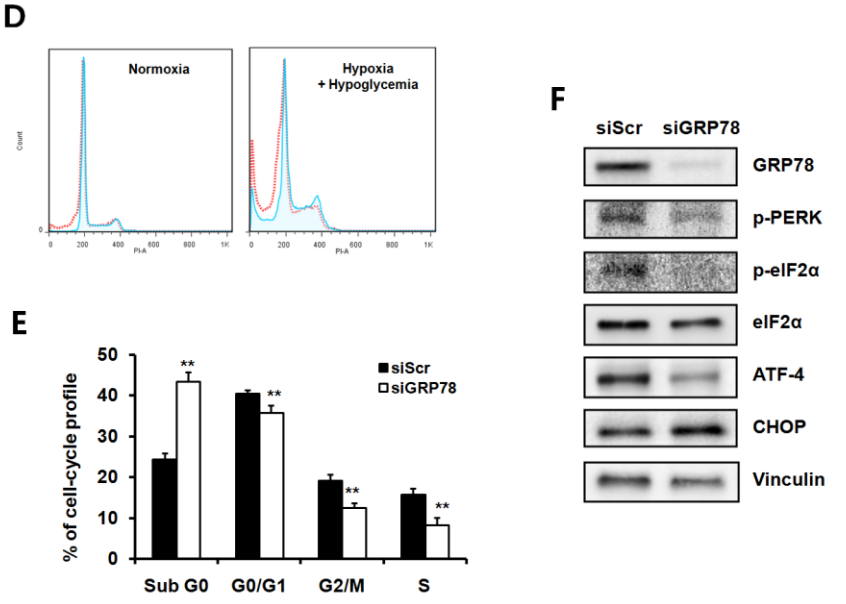


Figure 5. Changes in cell survival and apoptosis after knockdown of GRP78 in renal cell carcinoma cells.

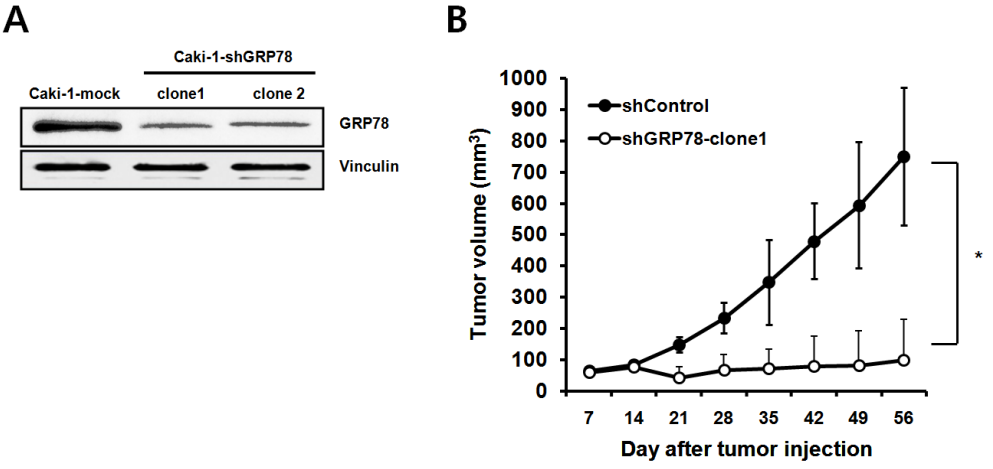
Transient knockdown of GRP78 using siGRP78 successfully suppresses the upregulation of GRP78 during hypoxia and hypoglycemia (A). Tumor cell survival is inhibited, * P <0.001 vs. scramble (B and C) and additional apoptosis is induced by GRP78 knockdown in Caki-1 cells, ** P <0.001 vs. siScr (D and E). Western blot of protein extracted from CaKi-1 cells treated with either siGRP78 or scrambled siRNA after hypoxic and hypoglycemic stress showed GRP78 knockdown inhibits phosphorylation of PERK and eIF2α, downregulates ATF-4, and induces CHOP (F).

6. In vivo knockdown of GRP78 induces tumor regression and enhances the anti-tumor effect of anti-angiogenic therapy in RCC xenografts

To confirm the effect of GRP78 inhibition on in vivo tumor growth, we developed Caki-1 cells stably expressing siGRP78 (Caki-1-shGRP78) or empty vector (Caki-1-Mock) and used these cells to established subcutaneous xenografts in mice (Fig. 6A). Tumors were successfully developed, and consistent tumor growth was achieved in the Caki-1-Mock xenografts. However,

tumor growth was not successfully achieved in the Caki-1-shGRP78 xenografts (Fig. 6B, data for clone 2 not shown). These results suggest that GRP78 has a vital role in tumorigenesis and tumor progression in RCC. Next, to determine whether targeting GRP78 has a therapeutic effect in RCC and whether inhibition of GRP78 makes RCC cells more vulnerable to anti-VEGF-targeted therapy, we developed tetracycline-inducible shGRP78-expressing Caki-1 cells and established subcutaneous xenografts using these cells in nude mice (Fig. 6C). Sunitinib was administered at the dosage of 40 mg/kg, which was effective for inhibition of tumor proliferation in previous study[16], and there was no significant side effect in mouse treated with sunitinib.

Mice treated with doxycycline to induce GRP78 knockdown had significantly reduced growth compared to the other two groups. The greatest growth inhibition was with the combination of sunitinib and GRP78 suppression (Fig. 6D and E). These results indicate that GRP78 inhibition enhances anti-tumor activity of anti-angiogenic therapy in vivo.



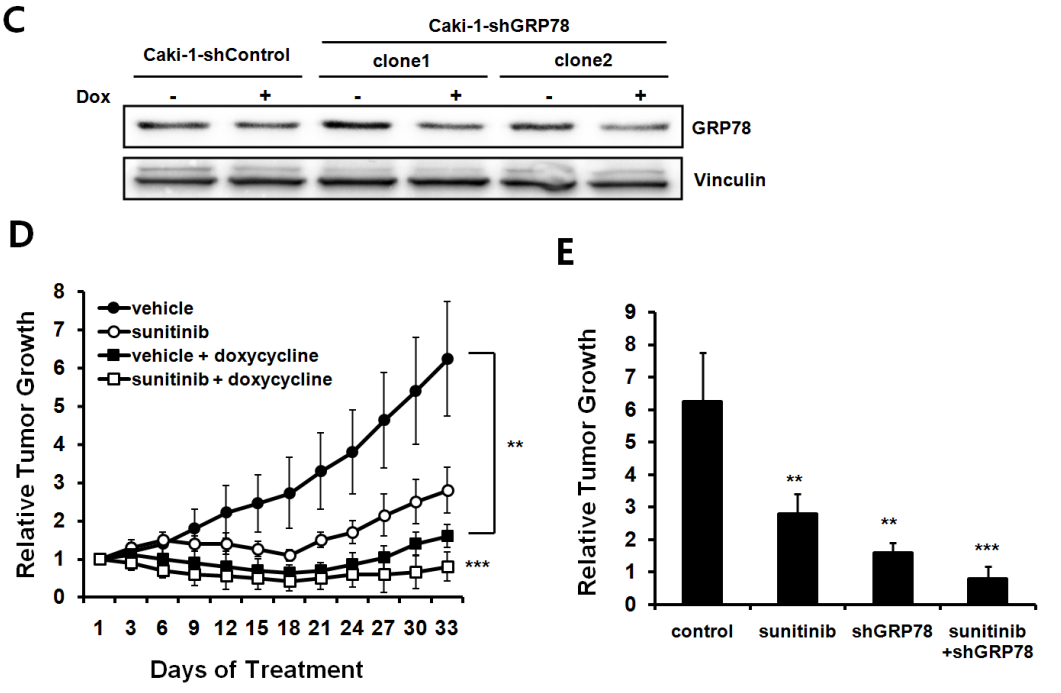


Figure 6. Effects of GRP78 knockdown on tumor growth in renal cell carcinoma xenografts.

Clones stably transfected with shGRP78 were developed (A). Knockdown of GRP78 suppresses tumor growth of renal cell carcinoma xenografts in nude mice as compared with control (12 mice in each group) * P <0.001 vs. shControl (B). Clones stably transfected with tetracycline-inducible shGRP78 were developed (C). After the tumors developed, combination of GRP78 knockdown with sunitinib (ie, combination of tetratcyline and sunitinib) shows strong suppression of tumor growth in mice, ** P < 0.001 vs. vehicle, *** P < 0.001 vs. sunitinib (D and E).

IV. DISCUSSION

VEGF-targeted anti-angiogenic therapy is a revolutionary treatment for RCC that has advanced treatment in advanced RCC beyond conventional chemo- and immunotherapy. In contrast to conventional cytotoxic therapy, anti-angiogenic therapy suppresses tumor cells by inducing tumor hypoxia and nutrient deprivation. Nevertheless, cancer cells eventually develop compensatory mechanisms to survive starvation and hypoxia. A response to anti-angiogenic therapy is the ER stress response, which allows tumor cells to survive the hypoxia, low-nutrients, or low-pH conditions during anti-angiogenic therapy. GRP78 plays a central role in this defense mechanism and is conservatively preserved in normal cells. However, a previous study showed that GRP78 heterozygosity impedes mammary tumor growth and inhibits tumor proliferation. GRP78 heterozygosity also promotes cancer apoptosis while mice exhibit normal growth, organ development, and antibody production, suggesting that GRP78 may be a good target candidate for cancer therapy[7]. Here, we showed that GRP78 expression was induced in RCC xenografts following anti-angiogenic therapy, and that GRP78 enhanced tumor growth in RCC cells and protected RCC cells from stress-induced apoptosis. Furthermore, GRP78 knockdown effectively inhibited tumor proliferation and induced apoptosis in RCC in vitro and in vivo by impeding UPR pathways.

The anti-tumor effects of anti-angiogenic agents are attributable to their ability to deprive tumors cells of vessels supplying oxygen and nutrients. Tumor cells can adapt to a low-oxygen environment owing to hypoxia-inducible factors (HIFs), which regulate the expression of more than 60 genes involved in angiogenesis, anaerobic glycolysis, and cell survival; the coordinated expression of these genes results in cellular adaptation to prolonged and acute hypoxia[17-19]. However, when the downstream effectors of HIFs are functionally blocked, hypoxia induces additional HIF-independent adaptive responses such as ER stress responses that contribute to increased survival under low-oxygen conditions. Thus, cells with a compromised ER stress pathway show significant sensitivity to ER stress created

by the deleterious effects of accumulated unfolded proteins in the ER[17, 20, 21].

As GRP78 is a master regulator of ER stress, UPR responses can be effectively downregulated by targeting GRP78. Hypoxic stress induces ER stress responses in a PERK- and eIF2α-dependent manner. UPR is orchestrated through activation of PERK, IRE1, and ATF6, all three of which are bound and sequestered by GRP78 under normal conditions[22]. When an unfolded protein load triggers the dissociation of GRP78 from these three sensors, UPR signaling is initiated. Activated PERK rapidly increases phosphorylation of the translation initiation factor eIF2α, which prevents the influx of additional nascent polypeptides to the ER[22] and upregulates genes that promote amino acid sufficiency and redox homeostasis[23-25]. Phosphorylation of eIF2α plays a central role in coupling the rate of protein synthesis to the cellular response to different types of stress and energy availability. A rapid and robust increase of eIF2α phosphorylation on ser51 is observed in response to hypoxia[26]. This modification correlates with a reduction in the protein synthesis rate and is not observed following other stresses such as low serum or genotoxic stress. The phosphorylation of eIF2α is independent of HIF1α status, as it also occurs to a similar extent in HIF1α−/− mouse embryonic fibroblasts [9, 27]. In the present study, the inhibition of GRP78 reduced the phosphorylation of PERK in RCC cells. In addition, the knockdown of GRP78 inhibited the activation of both PERK and eIF2α under both normoxic and hypoxic conditions, which suggests that GRP78 may be the best target for inhibiting the ER stress response.

CHOP is downstream of the PERK and eIF-2α phosphorylation cascade, and the activation of PERK and eIF2α induces CHOP accumulation, which plays a critical role in the switch from pro-adaptive to pro-apoptotic signaling[9]. CHOP promotes protein synthesis and oxidation in the stressed ER, thereby contributing to the induction of cell death. It modulates the Bcl-2 family of proteins and growth and arrest DNA damage inducible protein 34 (GADD34), causing damage to the mitochondrial membrane and the release of cytochrome C into the cytosol[28-30]. Our results show that the pro-apoptotic effects of GRP78 inhibition occur via CHOP activation subsequent to PERK and eIF2α activation in RCC

cells.

Activation of UPR exhibits a range of effects in altering the sensitivity or resistance of cancer cells in response to certain chemotherapeutic agents. Induction of UPR induces chemoresistance to both doxorubicin and 5-FU in breast cancer and hepatocellular carcinoma cells.[31,32] The use of GRP78 as a novel target to increase cell sensitivity to certain anticancer drugs has been suggested for malignant gliomas, melanoma, hepatocellular carcinoma, and diffuse large B-cell lymphoma.[33-36]

The inhibition of GRP78 halts the important defense mechanism required for survival in starving tumor cells and makes tumor cells more vulnerable to environmental stresses such as hypoxia and glucose deprivation. Combination therapy that stimulates ER stress-mediated apoptotic pathways and at the same time downregulates the induction of pro-survival GRP78 may substantially enhance drug efficacy. For this reason, targeting ER stress responses through GRP78 inhibition may be a reasonable approach for combination cancer therapy with VEGF-targeted anti-angiogenic agents. In addition to the protective role of GRP78 in cancer cells, GRP78 also promotes tumor proliferation. In the present study, overexpression of GRP78 increased the proliferation of RCC cells, and GRP78 knockdown had significant anti-tumor effects under normal conditions without stresses such as hypoxia and glucose deprivation. These results demonstrate that GRP78 plays a critical role in tumor proliferation and progression even in normal tumor environments, suggesting that targeting GRP78 may be an effective cancer treatment option as monotherapy.

V. CONCLUSION

Our study showed that the inhibition of GRP78 successfully enhanced the anti-tumor effect of sunitinib in RCC, indicating that GRP78 is an excellent target for combination therapy with anti-angiogenic treatments. Targeting ER stress responses may be a novel approach to enhance the therapeutic efficacy of anti-angiogenic agents and to delay the development of resistance.

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ABSTRACT(IN KOREAN)

신장암모델에서 소포체 샤페론 단백 GRP78 억제로 인한 수니티닙 항암효과의 강화

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김동석

신장암은 기존의 항암제에 반응률이 매우 저조한 암으로 최근 개발된 혈관억제 치료가 비교적 높은 치료 반응률을 보이고 있으나, 단기간 내의 내성 발현으로 인해 높은 반응률에도 불구하고 효과적인 생존률의 향상이 이루어지지 못하고 있다. 세포는 산소와 영양분이 부족한 상태에서 발생하는 이상에 대해 스스로 교정하고 조절함으로써 살아남을 수 있는 능력이 있는데 이중 핵심적인 역할을 하는 것이 소포체 스트레스 (ER stress) 반응이다. 세포는 소포체 내의 특정 단백질들을 통해 생명 유지에 필수적인 단백질을 제외한 일반 단백질에 대한 합성을 억제함으로써 이러한 저산소 스트레스에서 살아남게 되며 이를 접히지 않은 단백 반응 (unfolded protein responses, UPR) 또는 소포체 스트레스 반응이라고 하며, 이때 GRP78 단백이 중요한 역할을 한다. 본 연구에서는 소포체 샤페론 단백 GRP78의 발현을 조절함으로써 저산소 또는 저영양 상태에서 중요한 세포의 보호 작용인 소포체 스트레스가 신장암에서 가지는 분자생물학적 특성과 그 역할을 규명하고, 혈관억제치료에 미치는 영향을 연구하여 신장암 치료표적으로서의 가능성에 대해서 알아보고자 하였다.

본 연구를 통해 소포체 샤페론 단백 GRP78은 저산소, 저영양의 스트레스 환경에서 신장암을 보호하는데 중요한 역할을 함을 알 수 있었다. GRP78의 발현증가는 스트레스 환경에서 PERK, IRE1, ATF6 경로 활성화를 통해 신장암의 성장을 증진시키고 세포사멸로부터 신장암을 보호하였다. 또한 siRNA를 이용한 GRP78의 발현감소는 신장암의 성장을 억제하고 세포사멸을 유도하였으며, 신장암

모델에서 GRP78의 억제는 sunitinib의 항암효과를 보다 강화시키는 것을 보여주었다.
이 연구를 통해 소포체 스트레스 반응은 스트레스 환경에서 신장암의 성장과 세포사멸을 유도 또는 억제하는데 중요한 역할을 하고 있음을 확인하였고, 이는 GRP78이 신장암의 치료에서 단독으로 또는 혈관억제치료제와 함께 새로운 치료표적으로서 기능할 수 있는 가능성을 제시하고 있다.

핵심되는 말 : GRP78, 소포체스트레스, 신장암, 수니티닙