IMB-6G induces endoplasmic reticulum stress-mediated apoptosis in human nasopharyngeal carcinoma cells

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Abstract. IMB-6G is a novel N-substituted sophoridine acid that has been reported to have anticancer effects. The purpose of the present study was to investigate the effect and underlying mechanism of IMB-6G on human nasopharyngeal carcinoma (NPC) cells. The NPC cell line C666-1 was used in the present study and treated with different concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h. Subsequently, cell viability was determined using the Cell Counting kit-8 assay and cell apoptosis was analyzed by performing flow cytometry. The expression levels of genes and proteins in the current study were determined using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Results indicated that IMB-6G dose-dependently inhibited C666-1 cell viability and induced apoptosis. It was also revealed that IMB-6G induced apoptosis via inducing endoplasmic reticulum (ER) stress activation. Notably, IMB-6G administration enhanced the expression levels of Binding immunoglobulin protein and CCAAT-enhancer-binding protein homologous protein in C666-1 cells. Further analysis suggested that IMB-6G treatment activated inositol-requiring enzyme 1α (IRE1α) and PKR-like ER kinase (PERK) signaling pathways in C666-1 cells. In addition, silencing of IRE1α and PERK significantly reversed IMB-6G-induced cell growth inhibition and apoptosis. In conclusion, the present findings indicated that IMB-6G induced ER stress-mediated apoptosis through activating IRE1α and PERK signaling pathways. The present study suggests that IMB-6G may be a promising agent for NPC treatment.

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Introduction

As the most common malignant epithelial tumor of the head and neck in Southeast Asia and Southern China, nasopharyngeal carcinoma (NPC) seriously affects the quality of human life (1,2). Various factors, including genetic, environmental and viral factors are involved in the development of NPC (3-5). Notably, early detection of NPC remains difficult in the clinic because of a lack of NPC-identifiable symptoms and high metastatic potential. Approximately 75% of the patients were reported with the late stage NPC and local node metastases and/or distant metastasis at first diagnosis (6). Once recurrence or metastasis occurs following treatment, the prognosis of NPC is poor, which makes it the leading cause of fatality (7). Because of the frequent metastasis and poor prognosis, NPC has been identified as a highly malignant tumor (8). The standard approach for the treatment of NPC is chemoradiotherapy (9). Currently, although great progress has been made in the development of therapeutic strategies for NPC, the therapeutic efficacy remains unsatisfactory. Therefore, studies for novel treatment therapies for NPC are urgently required.

Sophoridine is a major bioactive component that is isolated from the traditional medicinal herb *Sophora alopecuroides* L (10). Studies have reported that sophoridine provides an anti-tumor effect against malignant trophoblastic tumors (11,12). As a novel N-substituted sophoridine acid derivative, IMB-6G has been indicated to have an anti-proliferation effect on several human tumor cells through inducing apoptosis and G_0/G_1 cell cycle arrest (13). Zhang *et al* (14) reported that IMB-6G induces endoplasmic reticulum (ER) stress-mediated apoptosis through the activation of inositol-requiring enzyme 1α (IRE1α) and PKR-like ER kinase (PERK) signaling pathways. Furthermore, IMB-6G also exhibits reasonable bio-availability and has been demonstrated to possess positive pharmacokinetic properties and exhibits good safety profile *in vivo* (13,15). However, to the best of our knowledge, no study has investigated the influence of IMB-6G in NPC cells.

The purpose of the present study was to investigate the anticancer activity and the underlying molecular mechanisms of IMB-6G against human NPC cells. The present findings investigated whether IMB-6G induces human NPC cell apoptosis by activating ER stress. Additionally, the study explored whether IRE1α- and PERK-mediated ER stress may be involved in IMB-6G-induced apoptosis.

Materials and methods

Cell culture and treatment. The NPC cell line C666-1 was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin/streptomycin solution at 37˚C in an atmosphere containing 5% CO_2. Cells were treated with various concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h at 37˚C as described previously (14) prior to experiments described below.

Cell transfection. The C666-1 cells were transfected with 50 nM IRE1α-small interfering (si)RNA (5'-GCGUCUUUU ACUACGUAAUCU-3'; Shanghai GenePharma Co., Ltd., Shanghai, China), 50 nM PERK-siRNA (cat no. sc-36213) or 50 nM control-siRNA (cat no. sc-37007; both Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Following 24 h of transfection, the cells were treated with or without 5 μM IMB-6G for 24 h prior to the subsequent experiments.

Cell viability detection. For cell viability detection, the Cell Counting kit-8 (CCK-8) assay was used. In brief, C666-1 cells were plated into a 96-well plate (5x10^3 cells/well) and incubated for 24 h at 37˚C. Subsequently, the cells were treated with various concentrations (0.000, 0.625, 1.250, 2.500, 5.000, 10.000 and 15.000 μM) of IMB-6G for 24 h. Each well was treated with 10 μl CCK-8 assay solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were subsequently incubated for a further 1 h at 37˚C. To assess the cell viability, the optical density at 570 mm was measured using a microplate reader.

Apoptosis analysis. To detect C666-1 cell apoptosis, an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (cat no. 45115; Cell Signaling Technology, Inc., Danvers, MA, USA) was utilized. C666-1 cells were treated with various concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h. C666-1 cells (5x10^5 cells per well) were subsequently labeled with Annexin V-FITC and propidium iodide per as the manufacturer's protocol. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze cell apoptosis, and data were analyzed using WinMDI (version 2.5; Purdue University Cytometry Laboratories; www.cyto.purdue.edu/flowcyt/software/Catalog.htm).

Western blot analysis. Total cellular protein from C666-1 cells was extracted using radioimmunoprecipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Nanjing, China). A bicinchoninic protein assay kit was used to detect protein concentration. Protein samples (25 μg per lane) were separated using 12% SDS-PAGE and then transferred to a polyvinylidene membrane. Following this, the membranes were blocked with 5% non-fat milk for 1 h at room temperature and blotted overnight at 4˚C with the following primary antibodies: Cleaved caspase-9 (cat. no. 20750; 1:1,000 dilution), cleaved caspase-3 (cat. no. 9579; 1:1,000 dilution), binding immunoglobulin protein (Bip; cat. no. 3177; 1:1,000 dilution), CCAAT-enhancer-binding protein homologous protein (CHOP; cat. no. 5554; 1:1,000 dilution), IRE1α (cat. no. 3294; 1:1,000 dilution; all Cell Signaling Technology, Inc.), p-IRE1α (ab48187; 1:1,000 dilution; Abcam, Cambridge, MA, USA), PERK (cat. no. 5683; 1:1,000 dilution), p-PERK (cat. no. 3179; 1:1,000 dilution), p-eukaryotic initiation factor 2α (eIF2α; cat. no. 3597; 1:1,000 dilution), eIF2α (cat. no. 5324; 1:1,000 dilution) and β-actin (cat. no. 4970; 1:1,000 dilution; all Cell Signaling Technology, Inc.). The membranes were subsequently incubated with the anti-rabbit IgG horseradish peroxidase-linked antibody (cat. no. 7074; 1:5,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 2.5 h. Protein bands were visualized using enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from C666-1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using PrimeScript RT Reagent kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. SYBR Premix Ex Taq II (Takara Bio Inc.) was used for qPCR analysis. Amplification conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 72˚C for 30 sec and 78˚C for 1.5 min. Samples were stored at 4˚C. GAPDH was used as an internal control. All primer sequences for PCR were listed in Table I. Relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. Experiments were performed in triplicate. Data were presented as the mean ± standard deviation. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was applied for all statistical analyses. One-way analysis of variance followed by a post hoc Tukey's test or a Student's t-test was used to analyze the differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IMB-6G inhibits C666-1 cell viability and induces cell apoptosis. To investigate the anticancer effect of IMB-6G on NPC, human NPC cells (C666-1) were treated with a variety of concentrations of IMB-6G (0.000, 0.625, 1.250, 2.500, 5.000, 10.000 and 15.000 μM) for 24 h, and assessed via CCK-8 assay analysis to measure C666-1 cell viability. Fig. 1A revealed the molecular structure of IMB-6G. As indicated in Fig. 1B, IMB-6G inhibited the cell viability of C666-1 cells in a dose-dependent manner. To determine whether cell apoptosis participates in IMB-6G-induced NPC cell death, C666-1 cell apoptosis was analyzed by flow cytometry following treatment with different concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h (Fig. 1C and D). Furthermore, western blot analysis indicated that IMB-6G promoted the expression levels of cleaved caspase-9 and cleaved caspase-3 (Fig. 1E). The findings suggested that IMB-6G does-dependently induced C666-1 cell apoptosis and inhibited cell growth.

IMB-6G activates ER stress in C666-1 cells. ER stress has been identified to serve an important role in regulating

Table I. Primer sequence for polymerase chain reaction.

Gene	Direction	Sequence (5'-3')
Bip	Forward	5'-TAGCGTATGGTGCTGCTGTC-3'
	Reverse	5'-TTTGTCAGGGGTCTTTCACC-3'
CHOP	Forward	5'-GAGGAGAGAGTGTTCAAGAAGG-3'
	Reverse	5'-TCTGGGAGGTGCTTGTGAC-3'
IRE1α	Forward	5'-TAGTCAGTTCTGCGTCCGCT-3'
	Reverse	5'-TTCCAAAAATCCCGAGGCCG-3'
PERK	Forward	5'-AGGACAGAGGGGACAGAGTTG-3'
	Reverse	5'-TAATGACCTTTTCTTCCCTGCTCC-3'
GAPDH	Forward	5'-CTTTGGTATCGTGGAAGGACTC-3'
	Reverse	5'-GTAGAGGCAGGGATGATGTTCT-3'

Bip, binding immunoglobulin protein; CHOP, CCAAT-enhancer-binding protein homologous protein; IRE1α, inositol-requiring enzyme 1α; PERK, PKR-like ER kinase.

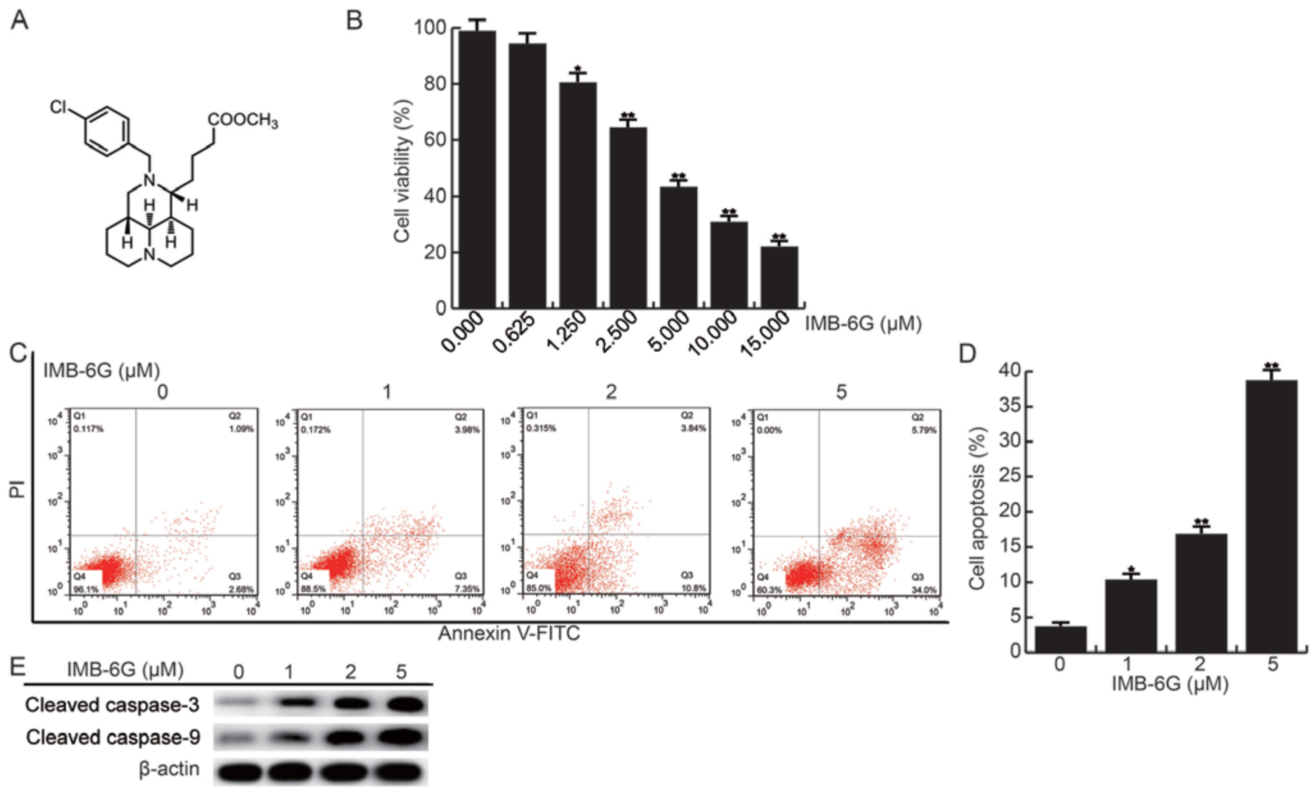

Figure 1. IMB-6G inhibits cell viability and induces apoptosis in C666-1 cells. (A) Structure of the IMB-6G molecule. (B) C666-1 cells were treated with various concentrations of IMB-6G (0.000, 0.625, 1.250, 2.500, 5.000, 10.000 and 15.000 μM) for 24 h and the cell viability was assessed using the Cell Counting kit-8 assay. (C) C666-1 cells were treated with various concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h and cell apoptosis was determined by flow cytometry. (D) Statistical analysis results of flow cytometric analysis of apoptosis were indicated. (E) Protein expression levels of cleaved caspase-9 and cleaved caspase-3 were measured using western blot analysis. Data were presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 μM IMB-6G. PI, propidium iodide; FITC, fluorescein isothiocyanate.

apoptosis (17,18). Thus, it was determined whether ER stress was involved in IMB-6G-mediated apoptosis in the present study. Following treatment with different concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h, the protein and mRNA expression levels of Bip and CHOP in C666-1 cells were detected by western blot analysis and RT-qPCR, respectively. Results indicated that IMB-6G dose-dependently increased the protein and mRNA expression levels of Bip and CHOP (Fig. 2).

IRE1α and PERK are involved in the IMB-6G-activated ER stress signaling pathway. To identify whether the ER stress signaling pathway is activated by IMB-6G, the phosphorylation of PERK, IRE1α and eIF2α was measured in C666-1 cells

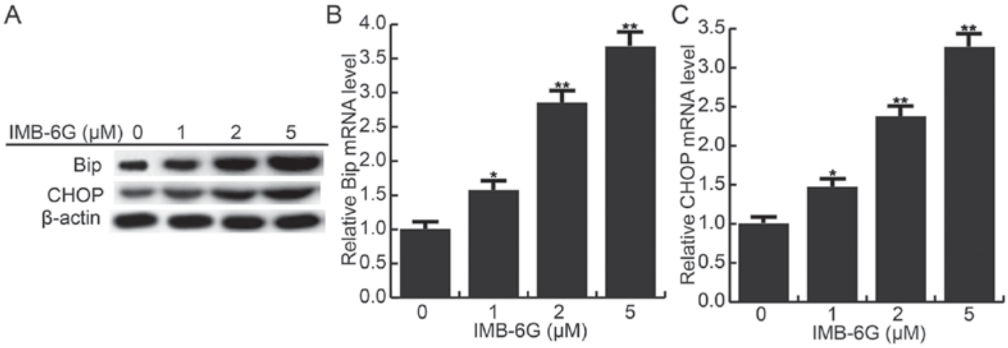

Figure 2. IMB-6G triggers ER stress in C666-1 cells. C666-1 cells were treated with various concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h and the expression levels of Bip and CHOP were measured by (A) western blot analysis and (B and C) reverse transcription-quantitative polymerase chain reaction, respectively. Data were presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 μM IMB-6G. Bip, binding immunoglobulin protein; CHOP, CCAAT-enhancer-binding protein homologous protein.

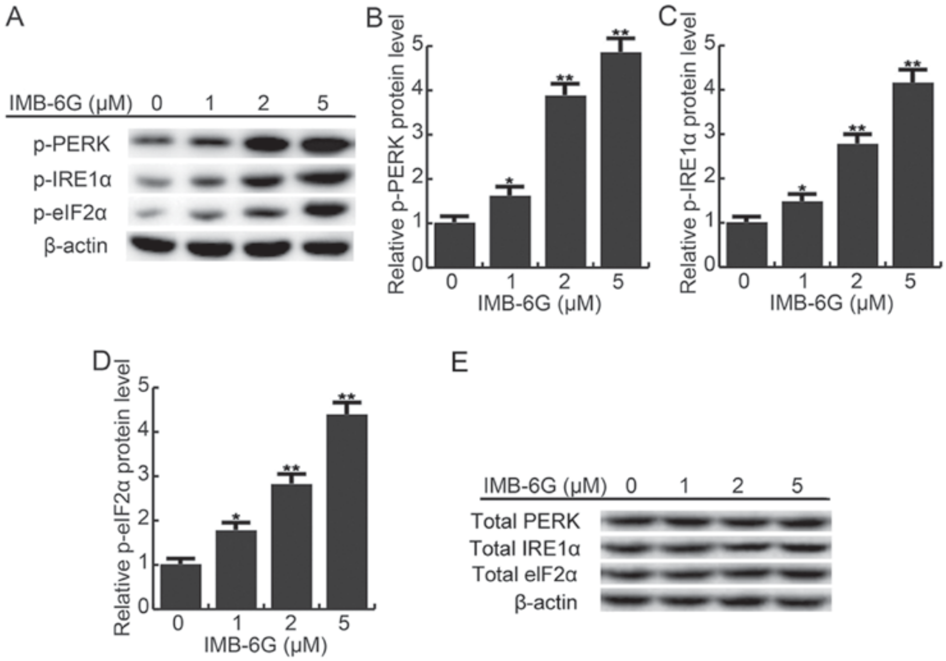

Figure 3. IMB-6G activates the ER stress signaling pathway via IRE1α and PERK. (A) C666-1 cells were treated with various concentrations of IMB-6G (0, 1, 2 and 5 μM) for 24 h and the phosphorylation of PERK (Thr980), IRE1α (Ser724) and eIF2α (Ser51) was measured by western blot analysis. (B-D) The protein expression levels of p-PERK (Thr980), p-IRE1α (Ser724) and p-eIF2α (Ser51) were analyzed. (E) The protein levels of of total PERK, total IRE1α and total eIF2α in C666-1 cells were measured by western blotting. Data were presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 μM IMB-6G. ER, endoplasmic reticulum; IRE1α, inositol-requiring enzyme 1α; PERK, PKR-like ER kinase; eIF2α, eukaryotic initiation factor 2α.

following treatment with IMB-6G at various concentrations of IMB-6G (0, 1, 2, 5 μM) for 24 h. Western blot analysis demonstrated that IMB-6G administration dose-dependently enhanced p-PERK, p-IRE1α and p-eIF2α expression levels (Fig. 3). Furthermore, the current study determined that IMB-6G administration exhibited no effect on the expression of PERK, IRE1α and eIF2α total protein in C666-1 cells.

IMB-6G induces C666-1 cell apoptosis via IRE1α and PERK pathways. To confirm whether the IRE1α and PERK signaling pathways are involved in IMB-6G-induced apoptosis of NPC cells, the effect of IRE1α or PERK inhibition on IMB-6G-induced C666-1 cell apoptosis was investigated. Following transfection with control-siRNA, PERK-siRNA or IRE1α-siRNA for 24 h, C666-1 cells were treated with 0 or 5 μM IMB-6G for another 24 h. The results demonstrated that IRE1α or PERK knockdown significantly reversed the IMB-6G-induced cell growth inhibition and apoptosis when compared with the control group (Fig. 4).

Discussion

NPC is considered to be relatively sensitive to chemotherapy due to the fact that >90% of NPCs are of the undifferentiated variety (19). Although some of the known therapies for NPC have demonstrated high efficacy, the survival of patients with advanced NPC has not been significantly improved (20). Therefore, there is an urgency to identify novel and efficient agents for the treatment of patients with advanced NPC. IMB-6G, a novel N-substituted sophoridinic acid derivative, has

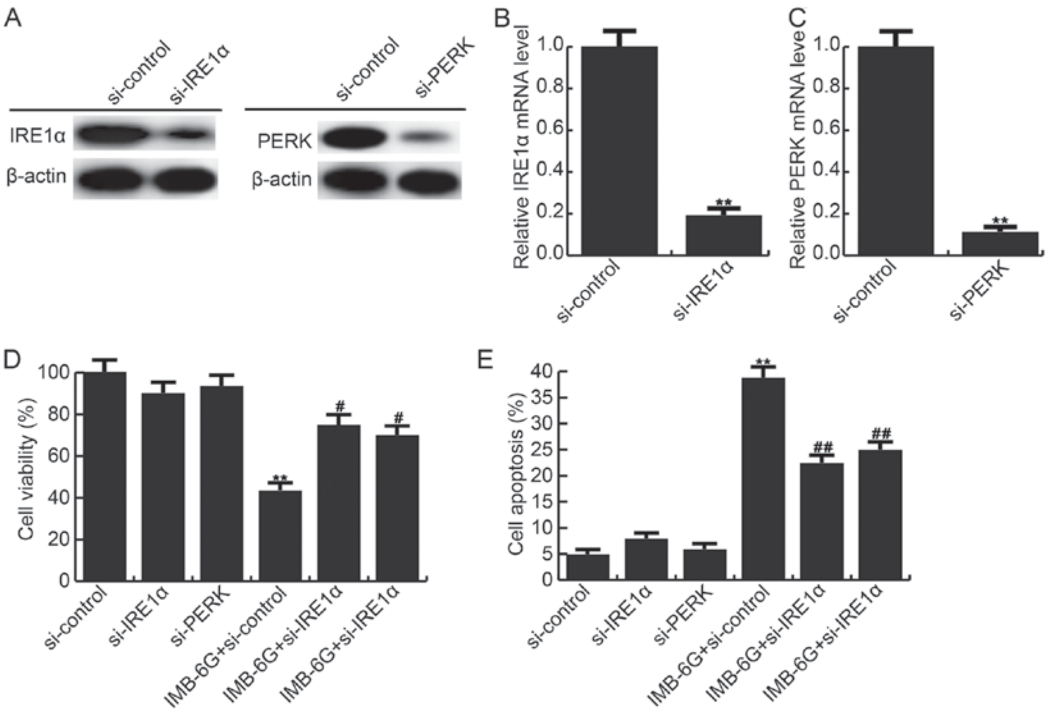

Figure 4. IRE1α and PERK signaling pathways are critical for IMB-6G-induced NPC cell viability inhibition and apoptosis. C666-1 cells were transfected with IRE1α-siRNA, PERK-siRNA or control-siRNA for 24 h and then treated with or without 5 μM IMB-6G for 24 h. (A) Protein expression levels of IRE1α and PERK were measured by western blot analysis. (B and C) mRNA expression levels of IRE1α and PERK were measured by reverse transcription-quantitative polymerase chain reaction. (D) Cell viability was assayed using a Cell Counting kit-8 assay. (E) Cell apoptosis was detected by flow cytometry. Data were presented as the mean ± standard deviation. **P<0.01 vs. si-control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. IMB-6G+si-control group. siRNA, small interfering RNA; IRE1α, inositol-requiring enzyme 1α; PERK, PKR-like ER kinase.

been demonstrated to have anti-tumor effect (13,14); however, little is known about its effect on NPC. In the present study, the effect of IMB-6G and any associated possible signaling pathways was investigated on human NPC C666-1 cells *in vitro*.

Firstly, the impact of IMB-6G on C666-1 cell viability was investigated. The results suggested that IMB-6G dose-dependently inhibited C666-1 cell viability and induced cell apoptosis, indicating IMB-6G induced cytotoxicity and apoptosis in human NPC cells. Mounting evidence has revealed that ER stress is critical in the regulation of apoptosis (17,18). ER serves an important role in regulating protein folding and trafficking, calcium homeostasis and lipid synthesis (21). Notably, a variety of factors can induce ER stress and trigger an adaptive response called the unfolded protein response (22). To investigate whether ER stress was involved in IMB-6G-mediated apoptosis, the ER stress responses in IMB-6G-treated NPC C666-1 cells were analyzed in the present study. The findings indicated that ER stress activation may be involved in the induced cytotoxicity of IMB-6G, as suggested by the increased expression levels of Bip and CHOP in IMB-6G-treated C666-1 cells. Subsequently, the signaling pathways that participated in the effect of IMB-6G on human NPC cells were explored. B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein-regulated Ca^{2+} release from the ER, IRE1α-mediated tumor necrosis factor receptor-associated factor 2 activation and PERK/eIF2α-dependent induction of the proapoptotic transcriptional factor CHOP (C/EBP homologous protein) are all associated with ER stress-mediated apoptosis (23-25). A previous study reported that IMB-6G induces ER stress-mediated apoptosis in human hepatocellular carcinoma cells by activating IRE1α and PERK signaling pathways (14). Therefore, it was investigated whether IRE1α and PERK signaling pathways were involved in ER stress-mediated apoptosis in human NPC C666-1 cells in the present study. The findings indicated that IMB-6G dose-dependently enhanced the expression levels of p-PERK and p-IRE1α, indicating the involvement of IRE1α and PERK signaling pathways in ER stress-mediated apoptosis in human NPC cells. In addition, it was revealed that IRE1α and PERK knockdown significantly reversed IMB-6G-induced cell viability inhibition and apoptosis, which indicated the activation of IRE1α and PERK signaling pathways served an important role in human NPC cell apoptosis induced by IMB-6G.

Taken together, the present study suggested that IMB-6G induced cytotoxicity and apoptosis in human NPC C666-1 cells through activating ER stress via the activation of IRE1α and PERK signaling pathways. The data indicated that IMB-6G is a potential anticancer agent that acts via inducing ER stress associated apoptosis in human NPC cells. Therefore, targeting ER stress by IMB-6G may be a promising treatment strategy for the treatment of NPC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP designed the current study. YP, YZ, and LG accessed and analyzed the data. JZ, BH and SZ interpreted results. All authors collaborated to write the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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