Abstract. Curcumin is a conventional Chinese medicine, which exerts a marked effect on various tumor types and suppresses tumor invasion. The present study analyzed the antineoplastic effects of curcumin on human nasopharyngeal carcinoma (NPC) cells and determined the effects of endoplasmic reticulum (ER) stress on curcumin-induced cytotoxicity. The Cell Counting Kit-8 assay examined the viability of SUNE1 and SUNE2 NPC cells. The Annexin V/propidium iodide staining technique was used to detect cell apoptosis and flow cytometry was used to examine cell cycle distribution. Western blotting and immunofluorescence were used to detect ER stress-associated molecules. Furthermore, the toxic effects of curcumin treatment alongside glucose-regulated protein 78 (GRP78) knockdown using small interfering (si)RNA, and treatment with the pan-caspase inhibitor Z-VAD-FMK and the protein kinase B (AKT) inhibitor MK-2206 were detected. The results demonstrated that curcumin markedly reduced cell viability, blocked cell cycle progression and induced apoptosis of human NPC cells. In addition, curcumin activated ER stress-associated proteins to participate in the apoptosis of human NPC cells. siRNA-induced knockdown of GRP78 may be able to strengthen the toxic effects of curcumin through mediating the AKT signaling pathway. These findings indicated that downregulation of GRP78 promoted the therapeutic effects of curcumin on NPC cells. The present study identified a potential, novel therapeutic method for the treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common primary malignancy of the nasopharynx, which frequently occurs in South China and Southeast Asia (1). The main treatment strategy for NPC is a combination of radiotherapy and chemotherapy; however, due to distant metastasis and resistance to treatment, the clinical prognosis of NPC is poor (2). In addition, numerous therapeutic measures can cause serious side effects and lead to multidrug resistance (3). Therefore, it is necessary to identify a novel, safe and effective method to treat NPC. At present, for the prevention, inhibition or delay of carcinogenesis, treatment with natural, synthetic or biological chemicals is regarded as being able to induce relatively effective chemoprevention (4).

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is present in turmeric (Curcuma longa), and has been used to treat various diseases, including allergies, coryza, cough, anorexia, rhinitis and hepatic conditions, in Asia for centuries (5). In addition, curcumin is able to suppress proliferation and promote apoptosis in various malignant diseases (6-9). However, the functions and effects of curcumin on NPC cells require further research. Some specific functions that have been detected in other tumor models include induction of apoptosis via c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathway regulation (10), endoplasmic reticulum (ER) stress (11) and autophagy (12).

The ER is an organelle, which can fold and synthesize transmembrane, intraorganelar and secretory proteins, and accumulate intracellular calcium. Disturbance of ER homeostasis leads to ER stress and the subsequent accumulation of unfolded or misfolded proteins; this is known as the unfolded protein response (UPR). The UPR induces a series of signaling pathways to maintain ER homeostasis via the upregulation of molecular chaperones that can accelerate immunoglobulin folding and protein synthesis attenuation (13). Three ER transmembrane receptors inhibit the three signaling pathways that comprise the UPR; inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 and eukaryotic translation initiation factor 2α kinase 3 (14). Initially, UPR signaling serves a crucial role in restoring ER homeostasis; however,
continuous or prolonged UPR results in cell apoptosis via
caspase-12, caspase-4 and B-cell lymphoma 2 (Bcl-2). The
present study aimed to investigate the effects of the UPR
on the toxic activity of curcumin on NPC cells. The effects
of interference in the UPR pathway on the toxic effects of
curcumin on human NPC cells were also analyzed.

Materials and methods

Cell culture. SUNE1 and SUNE2 NPC cells were purchased
from the American Type Culture Collection (Manassas, VA,
USA). These cells were cultured in Dulbecco's modified
Eagle's medium (HyClone; GE Healthcare Life Sciences,
Logan, UT, USA) or RPMI-1640 medium (Gibco; Thermo
Fisher Scientific, Inc., Waltham, MA, USA) supplemented
with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific,
Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco;
Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in
an atmosphere containing 95% air and 5% CO₂.

Reagents and antibodies. Curcumin, Z-VAD-FMK (Z-VAD)
and MK-2206 were purchased from Sigma-Aldrich; Merck
KGaA (Darmstadt, Germany). Primary antibodies against
GRP78 (cat. no. ab21685), GAPDH (cat. no. ab37168),
cleaved poly (ADP-ribose) polymerase (PARP; cat.
no. ab32561), caspase-3 (cat. no. ab90437), caspase-9 (cat.
no. ab25758) and caspase-12 (cat. no. ab62484) were
obtained from Abcam (Cambridge, UK). Antibodies against
CCAT-enhancer-binding protein homologous protein
(CHOP; cat. no. 2895), IRE1 (cat. no. 3294), phosphorylated
(p)-eukaryotic initiation factor 2α (eIF2α; cat. no. 9721),
Bcl-2 (cat. no. 2872), protein kinase B (AKT; cat. no. 9272)
and p-AKT (Ser473) (cat. no. 9271) were purchased from Cell
Signaling Technology, Inc. (Danvers, MA, USA).

Measurement of cell viability. Cell viability was analyzed
using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular
Technologies, Inc., Kumamoto, Japan). Initially, cells
(5x10³/well) were cultured in culture medium and were
incubated into 96-well plates. After 24 h, 0, 10, 20, 40 and
80 µM curcumin was added to each well and the cells were
cultured at 37°C for a further 24 h. The original culture
medium was replaced with a mixture of fresh medium and
CCK-8 solution (10 µl). The optical density (OD) index of the
cells was measured at 450 nm after culturing at 37°C for 2 h.
The percentage of inhibition of cell activity was calculated
according to the following formula: (control OD value-experi-
ment OD value)/control group OD value x100%.

Cell cycle analysis by flow cytometry. Human NPC cells were
cultured in the aforementioned culture medium. Subsequently,
when the cells reached 50% confluence, they were treated with
80 µM curcumin at 37°C for 24 h and were collected in order to
analyze the cell cycle. Briefly, 5x10⁴ cells were incubated with
DAPI and RNase A (Thermo Fisher Scientific, Inc.) for
30 min at room temperature, and the cells were then suspended
in 0.5 ml propidium iodide (PI; BD Biosciences, San Jose, CA,
USA) solution and incubated for 30 min at room tempera-
ture in the dark, according to the manufacturer's protocol.
Subsequently, cell cycle distribution was analyzed using
flow cytometry [fluorescence-activated cell sorting (FACS)
analysis; BD FACSDiva software v8.0.1; BD Biosciences].

Apoptotic analysis by flow cytometry. Cells (4x10⁵/well) were
seeded in the aforementioned culture medium in 6-well plates
and were incubated at 37°C for 24 h. Subsequently, cells were
treated with fresh medium containing the required concentra-
tions of curcumin (0, 20, 40 and 80 µM), or the cells were
treated with 50 µM Z-VAD, followed by treatment with or
without 80 µM curcumin at 37°C for 24 h. Cells were then
stained using the Annexin V-fluorescein isothiocyanate
(FITC) apoptosis detection kit (BD Biosciences). According
to the manufacturer's protocol, cells were treated for 15 min
with a mixture of Annexin V (5 µl) and PI (5 µl) at room
temperature. Subsequently, the stained cells were observed
by flow cytometry (FACS analysis; BD FACSDiva software
v8.0.1, BD Biosciences).

Western blot analysis. Cells were treated with either curcumin
(0-80 µM) or MK-2206 (5 µM) alone or in combination for
24 h. Alternatively, cells underwent GRP78 knockdown by
transient transfection with small interfering (si)RNA (20 nM)
for 24 h, after which the transfected NPC cells were treated
with or without curcumin (80 µM) in complete medium at
37°C for 24 h. The cells were then lysed in cold radioimmuno-
precipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA)
supplemented with 1 nM phenylmethylsulfonyl fluoride,
and were centrifuged at 12,000 x g for 10 min at 4°C. The
concentration of proteins extracted from cells was determined
using a bicinchoninic acid assay (Bio-Rad Laboratories, Inc.,
Hercules, CA, USA). Total proteins (20-25 µg) were separated
by 8-15% SDS-PAGE and transferred onto a polyvinylidene
fluoride membrane (EMD Millipore, Billerica, MA, USA).
The membrane was then blocked in a mixture of 5% skimmed
milk and PBS containing 0.1% Tween-20 for 2 h at room
temperature, and was incubated with primary antibodies at
4°C overnight [1:1,000 dilutions for GRP78, cleaved PARP,
caspase-3, caspase-9, caspase-12, CHOP, IRE1, p-eIF2α, Bcl-2,
AKT, p-AKT (Ser473) and GAPDH]. Membranes were then
incubated with horseradish peroxidase-conjugated secondary
antibodies (cat. nos. 7074 and 7076; 1:2,000; Cell Signaling
Technology, Inc., for 1 h at room temperature, followed by
visualization with enhanced chemiluminescence immuno-
binding-lotting detection reagents (EMD Millipore). Protein band
intensities were semi-quantified by densitometric analysis using
ImageJ software 1.6.0_20 (National Institutes of Health,
Bethesda, MD, USA).

Immunofluorescence assay. A total of 1x10⁵ human NPC cells
were plated in each well of 6-well chamber slides and were
treated with curcumin (80 µM) at 37°C for 24 h. PBS was
used to wash the cells, and they were then immobilized and
permeabilized with 4% paraformaldehyde and 0.1% Triton
X-100 for 15 min at room temperature. Immunofluorescence
staining was then conducted. Initially, the processed human
NPC cells were stained with primary antibodies (1:1,000 for
GRP78; 1:3,000 for CHOP) for 2 h at room temperature,
and were then cultured with secondary antibodies conjugated
to Alexa Fluor 488 (green) (1:200) or Alexa Fluor 546 (red)
(1:200) (cat. nos. A28175 and A-11071; Invitrogen; Thermo
Curcumin reduces the viability of human NPC cells. In order to investigate the role of curcumin in the survival of NPC cells in vitro, SUNE1 and SUNE2 cells were exposed to various concentrations of curcumin (0-80 µM) for 1 day, and inhibition of cell proliferation was evaluated by CCK-8. As shown in Fig. 1, inhibition of NPC cell viability increased with increasing concentrations of curcumin, from 13.58% inhibition following treatment with 10 µM curcumin to 40.60% inhibition following treatment with 80 µM curcumin in SUNE1 cells, and from 15.16% inhibition following treatment with 10 µM curcumin to 43.27% inhibition following treatment with 80 µM curcumin in SUNE2 cells.

Curcumin induces NPC cell apoptosis and cell cycle arrest. In order to determine the role of curcumin in apoptotic cell death, SUNE1 and SUNE2 cells were exposed to various concentrations of curcumin (20-80 µM) for 24 h. Apoptotic cell death was analyzed using the Annexin V/PI analysis. As shown in Fig. 2A-D, early apoptosis of SUNE1 cells was markedly increased from 1.49 to 17.45% and late apoptosis of SUNE1 cells was increased from 0.72 to 21.38% following exposure to 80 µM curcumin for 24 h. In addition, early and late apoptosis increased from 1.16 to 25.12% and 2.23 to 15.93% in 80 µM curcumin-treated SUNE2 cells, respectively. As shown in Fig. 2E, cell cycle distribution of curcumin-treated SUNE1 and SUNE2 cells was determined. The majority of curcumin (80 µM)-treated NPC cells were arrested at G0/G1 phase; the percentage of cells in G0/G1 phase was increased to 68.85 and 70.88% in SUNE1 cells and SUNE2 cells, respectively. These data indicated that curcumin can induce cell death via apoptosis.

Curcumin induces ER stress in human NPC cells. To determine the effects of curcumin on ER stress in human NPC cells, specific ER-associated proteins were detected. Western blot analysis demonstrated that IRE1, p-eIF2α, CHOP and GRP78 were increased following curcumin treatment (Fig. 3A-D). To ascertain whether curcumin can increase the expression of markers that are associated with ER stress in NPC cells, CHOP and GRP78 were analyzed by immunofluorescence staining. As shown in Fig. 3E, immunofluorescence staining of CHOP and GRP78 was markedly increased following curcumin (80 µM) treatment in SUNE1 and SUNE2 cells. Previous studies have reported that prolonged ER stress can active caspase-12, and caspase-12 has an important influence on inducing cell death (15,16). In order to analyze the effects of curcumin on ER stress and toxicity, and to investigate the underlying mechanisms, western blotting was used to detect the expression of the caspase-12 protein in NPC cells. As shown in Fig. 4A-D, compared with in the control group, activation of caspase-12 was increased in curcumin-treated SUNE1 and SUNE2 cells. Furthermore, the expression levels of the anti-apoptotic protein Bcl-2 were significantly decreased (Fig. 4A-D). These results indicated that ER stress may induce apoptosis.

GRP78 knockdown enhances curcumin-induced apoptosis of human NPC cells. GRP78, which is the most important marker of the UPR, has been reported to be associated with chemoresistance (17,18). siRNA was used to determine the effects of GRP78 knockdown on curcumin-induced apoptosis of SUNE1 and SUNE2 cells. As shown in Fig. 5A, following GRP78 siRNA transfection, in cells that were treated with curcumin, cell viability was significantly reduced. In addition, GRP78 siRNA transfection significantly inhibited the protein expression levels of GRP78, improved PARP, caspase-3 and caspase-9 cleavage (Fig. 5B and C), and increased cell apoptosis (Fig. 6A and B) of curcumin-treated SUNE1 and SUNE2 cells. Subsequently, the pan-caspase inhibitor Z-VAD was used to investigate the role of curcumin in cell death. As shown in Fig. 6C and D, a marked reduction in cell death was detected in SUNE1 and SUNE2 cells pretreated with Z-VAD. This finding indicated that curcumin-mediated apoptosis may be associated with caspase activation.
GRP78 knockdown reduces AKT activity in human NPC cells. The AKT pathway is important in the modification of cell growth and proliferation (19). Previous studies have reported that the GRP78 signaling pathway can regulate the AKT signaling pathway (20,21). Therefore, the present study aimed to determine whether AKT was involved in GRP78-mediated cell death during curcumin treatment. As shown in Fig. 7A and B, treatment of SUNE1 and SUNE2 cells with curcumin (80 µM) significantly suppressed AKT phosphorylation, whereas total AKT was only slightly altered. Knockdown
of GRP78 via siRNA alongside curcumin treatment led to a marked reduction in AKT phosphorylation compared with cells treated with curcumin only. To further investigate the effects of GRP78 expression and AKT phosphorylation, MK-2206, a chemical inhibitor of AKT, was used to treat cells prior to treatment with curcumin. As shown in Fig. 7C and D, treating SUNE1 and SUNE2 cells with MK-2206 reduced activation of AKT. Furthermore, MK-2206 markedly
decreased the expression levels of GRP78 (Fig. 7C and D). These results indicated that GRP78 knockdown may reduce AKT activity.

Discussion

NPC is a common cancer of the nasopharynx, with a five year survival rate of 40-70% (22). Numerous patients with NPC experience recurrence or metastasis due to resistance to standard therapy with radiation (23). Therefore, it is very important to identify novel treatment modalities. Curcumin, which is a polyphenolic dienone, has been demonstrated to induce apoptosis in several human malignancies (24-27); however, the mechanisms underlying the suppressive effects of curcumin on NPC remain to be completely explored. The present study demonstrated that curcumin included ER stress, cell cycle arrest and apoptosis of human NPC cells. Knockdown of GRP78 by siRNA was able to significantly enhance curcumin-induced NPC cell apoptosis.

Previous studies have reported that ER stress is not only conducive to cell survival, but also induces apoptosis in various cell types (28,29). The aim of the UPR is to reinstate appropriate ER homeostasis; however, if ER stress perseveres, the UPR signaling pathways can trigger apoptosis. Therefore, the balance between the survival signal and the apoptosis signal determines the survival and apoptosis of cells. GRP78 is an ER molecular chaperone, which is important in folding and assembling proteins (30). Previous research has indicated that constitutive overexpression of GRP78 is associated with chemoresistance in cancer treatment (31), whereas knockdown of GRP78 can enhance chemosensitivity in several tumor cells (18,32,33). According to the present study, treatment of human NPC cells with curcumin led to ER stress, and curcumin-induced ER stress in SUNE1 and SUNE2 cells was associated with the upregulation of GRP78. In addition, silencing GRP78 using siRNA significantly enhanced the cytotoxic and apoptotic effects of curcumin in SUNE1 and SUNE2 cells. These data revealed that GRP78 may be crucial in protecting human NPC cells from curcumin-induced apoptosis. Therefore, downregulation of GRP78 may markedly improve the sensitivity of human NPC cells to curcumin.

Activation of the AKT signaling pathway is crucial in modifying cell proliferation and motility in various types of cancer; therefore, inhibition of AKT phosphorylation could suppress tumor cell growth and proliferation (34,35). In addition, it has been demonstrated that the AKT signaling pathway is associated with chemoresistance and maintaining GRP78 expression (36,37). The present results revealed that GRP78 knockdown by siRNA significantly inhibited the phosphorylation of AKT in curcumin-treated cells. Furthermore, inhibiting AKT activity using the chemical inhibitor MK-2206 prevented curcumin-mediated GRP78 induction, and suppressed AKT activity, thus suggesting that GRP78 may function in NPC to restrict chemotherapeutic-induced cytotoxicity via regulating the AKT signaling pathway.

In conclusion, this study suggested that curcumin may inhibit the viability and promote the apoptosis of human NPC cells. Downregulation of GRP78 may further enhance curcumin-induced SUNE1 and SUNE2 cell apoptosis. These findings are promising; however, further research is required to develop novel therapeutic strategies for human NPC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.
Authors’ contributions

XY performed experiments and analyzed data; XZ designed the research, interpreted data and edited the manuscript; LS wrote the manuscript and analyzed the data; LY analyzed the data and reviewed the manuscript; HW interpreted data and reviewed the manuscript; YW interpreted data and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.
Figure 6. Downregulation of GRP78 strengthens the effects of curcumin on apoptosis of human nasopharyngeal carcinoma cells. (A and B) Flow cytometry was used to study apoptotic cells stained with PI and Annexin V-FITC. Quantitative examination of early and late apoptotic populations. (C and D) Effects of Z-VAD and curcumin on apoptosis of SUNE1 and SUNE2 cells. Following pretreatment with 50 μM Z-VAD, NPC cells were treated with or without 80 μM curcumin. Apoptotic cells were detected by flow cytometry using PI and Annexin V-FITC staining. Data are presented as the means ± standard deviation of three independent tests. *P<0.05, **P<0.01. FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, small interfering RNA; Z-VAD, Z-VAD-FMK.

Figure 7. AKT and GRP78 signaling may have a role in human nasopharyngeal carcinoma cells, as determined by GRP78 knockdown and AKT inhibition. (A and B) GRP78 knockdown by transient transfection of siRNA (20 nM) for 24 h, followed by treatment with or without curcumin (80 μM) and western blotting was conducted to detect p-AKT and t-AKT. GAPDH was used as an internal control. The blots represent the results of at least three independent tests. Band density in each assay was measured and normalized to that of GAPDH. Data are presented as the means ± standard deviation of three independent tests. "P<0.01 vs. the control group; "P<0.05, ""P<0.01 vs. curcumin-treated cells. (C and D) SUNE1 and SUNE2 cells were exposed to curcumin (80 μM) for 24 h in the presence or absence of MK-2206 (5 μM). GRP78 and p-AKT expression was evaluated by western blotting. GAPDH was used as an internal control. The blots represent the results of at least three independent tests. Band density in each assay was measured and normalized to that of GAPDH. Data are presented as the means ± standard deviation of three independent tests. *P<0.05, **P<0.01 vs. curcumin-treated cells; *P<0.05 vs. MK-2206-treated cells. AKT, protein kinase B; GRP78, glucose-regulated protein 78; p-, phosphorylated; siRNA, small interfering RNA; t-, total.
Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


