Original Paper

Mitofusin-2 Triggers Cervical Carcinoma Cell HeLa Apoptosis via Mitochondrial Pathway in Mouse Model

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Key Words

Mfn2 • HeLa • Apoptosis • Bax • Bcl-2 • Mitochondrial pathway

Abstract

Background/Aims: Cervical carcinoma continues to be one of the most dangerous cancer types, and more effective therapies are urgently needed for cervical carcinoma treatment. Mitochondria-associated Mitofusin 2 has influence on the progression of many cancers. In the current study, we aimed to focus on the cell apoptotic effects of Mfn2 on cervical carcinoma HeLa cells *in vitro* and to try to explore its underlying mechanisms. Moreover, we investigated the anticancer potential of Mfn2 in a cervical carcinoma mouse model. **Methods:** Adenovirus-Mfn2 (Adv-Mfn2) was used to deliver mfn2 into HeLa cells and tumour tissues in a nude mouse model. CCK-8, TUNEL assay, Western blot and immunohistochemical staining were performed to detect the effects of Mfn2. The mRNA level of Mfn2 was determined by quantitative real-time PCR (qRT-PCR) analysis. The effect of Mfn2 on cell apoptosis was investigated by flow cytometry. Flow cytometry was used to assess the change of the mitochondrial membrane potential of the cells treated with JC-1 assay. Mfn2, Bax, Bcl-2, cytochrome c, cleaved caspase-3, and cleaved caspase-9 protein levels were analysed by Western blot. **Results:** Data from CCK-8 and flow cytometry showed that Mfn2 could inhibit proliferation and induce apoptosis in a dose- and time-dependent manner in HeLa cells. JC-1 test results revealed that the membrane potential of the mitochondria decreased in a dose-dependent manner in HeLa cells after Adv-Mfn2 treatment. The data from Western blot confirmed that higher cytosolic amounts of cytochrome c with increasing doses of Adv-Mfn2 signified the onset of the intrinsic apoptotic pathway. Levels of cleaved caspase-3 and cleaved caspase-9 increased in HeLa cells with Adv-Mfn2 treatment. We also found significant increases in the Bax level and a decreased Bcl-2 level with Adv-Mfn2 treatment. We further confirmed that Mfn2 could significantly inhibit the growth of the cervical tumour in the xenografted cervical carcinoma mouse model. After a 9-day-treatment, the tumours of the Adv-mfn2 group were inhibited and induced into apoptosis. The results demonstrated that the overexpression of Mfn2 could not only increase the levels of Bax and Bid in cervical tumour cells but also decrease the phosphorylation of Bad

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and the expression of Bcl-2. **Conclusion:** These studies suggested that the overexpression of Mfn2 could trigger cervical tumour apoptosis *in vitro* and *in vivo*, which was related to the mitochondrial pathway, and may provide a new treatment target for cervical carcinoma.

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Introduction

Based on the GLOBOCAN estimates, approximately 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide. Over the years, the burden has shifted to less developed countries, which currently account for approximately 57% of the cases and 65% of the cancer deaths worldwide [1]. Targeting single molecular abnormalities or cancer pathways, which could modestly affect survival in some cancers, has achieved good clinical response [2]. Capabilities acquired as cancer hallmarks, including resisting cell death and so on, have stood the test of time as being integral components of most forms of cancer [3, 4]. These hallmarks contain many treatment targets, like the “apoptotic trigger” that conveys signals between the regulators and effectors, which is controlled by counterbalancing pro- and anti-apoptotic members of the Bcl-2 family of regulatory proteins [5].

The mitochondria controls many vital cellular parameters, such as the balance between mitochondrial fission and fusion, which affects the morphology of the mitochondria and also helps the mitochondria change the cytosolic localization, like accumulating where high amounts of ATP required or where Ca^{2+} signalling needs to be regulated [6, 7]. Fusion of the mitochondria can attenuate stress by mixing the contents of partially damaged mitochondria as a form of complementation, and fission could not only lead to new mitochondria but also contribute to quality control by removing the damaged mitochondria [8-10].

The mitofusins (Mfn1 and Mfn2) associate with mitochondria and alter the mitochondrial morphology when expressed by transient transfection in tissue culture cells, which are homologs of the *Drosophila* protein, fuzzy onion (Fzo), and have been shown to affect the mitochondrial morphology when overexpressed yet play a reciprocal role in shaping the mitochondrial morphology in mammals [6, 11].

Mitofusin-2 (Mfn2) participates in mitochondrial fusion and contributes to the maintenance of the mitochondrial network [12-15]. The domain structure of the Mfn2 protein contains a p21^{ras} signature motif, a GTP binding site motif, a possible PKA/PKG phosphorylation site, and a hydrophobic transmembrane domain [16]. An internal region, including a predicted bipartite transmembrane domain, is sufficient to target Mfn2 to the mitochondria and requires hydrophobic residues within the transmembrane domain [11].

Mfn2 is involved in the pathogenesis of hyper-proliferative diseases and in cancers as well, which makes Mfn2 a powerful cell proliferation suppressor [13, 16-18]. Studies on vascular hyper-proliferative disorders have proven that Mfn2 functions as a cell proliferation suppressor in serum-evoked vascular smooth muscle cells (VSMCs) in culture and in blocked balloon injury-induced neointimal VSMCs, whereas the over-expression of Mfn2 could markedly suppress restenosis in rat carotid arteries [16].

Notably, previously unappreciated effects of Mfn2 in promoting apoptosis have immediately been noticed [13, 19]. The Mfn2 gene is localized in the human chromosome 1p36.22 [6, 16], whereas chromosome 1 aberrations are related to various human cancers, such as neuroblastoma, breast cancer and cervical cancer, which may suggest the existence of tumour suppressor genes in this region [20, 21]. Recently, the pro-apoptotic and anti-proliferative effects of Mfn2 in different types of cancers have been studied, including liver, breast and urinary bladder cancers [22-24]. Mfn2 is frequently lost in breast cancer, which has influence on breast cancer progression by a new mitochondria reprogramming pathway [17], and the PI3K-AKT-mTOR pathway plays an important role in the activation-induced downregulation of Mfn2 and the subsequent proliferation of resting human T cells [25]. In our early study, we found that the PTD4-apoptin fusion protein could induce HeLa cell apoptosis and upgrade Mfn2 expression [26]. However, the effects of Mfn2 in cervical cancer have rarely been investigated, and the underlying mechanism is worth researching.

In the current study, we focused on the cell apoptotic effect of Mfn2 on the cervical carcinoma HeLa cells *in vitro* and tried to explore the underlying mechanisms. Moreover, we investigated the anticancer potential of Mfn2 *in vivo* in a cervical carcinoma mouse model.

Materials and Methods

Antibodies

Antibodies used were as follows: Anti-Mfn2 (Abcam, USA) directed against the Mfn2 protein, p53 (Cell Signalling Technology, USA), cleaved caspase-3 (Cell Signalling Technology, USA), cleaved caspase-9 (Cell Signalling Technology, USA), Bcl-2 (Affinity Biosciences), Bid (Affinity Biosciences), Bax (Cell Signalling Technology, USA), p-Bad (Cell Signalling Technology, USA) and cytochrome c (Cell Signalling Technology, USA). GAPDH (Santa Cruz, USA) and β -actin (Tianjin Sungene Biotech, China) antibodies were used as internal loading controls.

Construction of mfn2 expression adenoviral vector

Rno-mfn2 precursor DNA (*Homo sapiens* (human), Gene ID: 9927) was synthesized by Genechem (Shanghai, China). The adenovirus expressing mfn2 (Adv-Mfn2), or controlling the control adenovirus expression (Adv-control) was generated using the AdMax system (Microbix Biosystems, Canada) according to the manufacturer's protocols. These resulting adenoviruses were further packaged and amplified in HEK293 cells and purified using CsCl banding. Viral titre was routinely concentrated to nearly 1×10^9 PFU/ml as determined by a plaque assay.

Cell culture

The human cervical carcinoma cell line, HeLa, was purchased from the Chinese Culture Tissue Collection Centre and cultured in RPMI 1640 (Thermo Fisher Scientific, Beijing, China) supplemented with 10% foetal bovine serum (FBS, Gibco BRL Life technologies, USA) under a humidified 5% CO₂ atmosphere at 37 °C. Cells were checked routinely and trypsinized until they reached 80-90% confluency.

CCK-8 test

The cell viability was measured via a cell counting kit-8 (CCK-8 kit, Dojindo Molecular Technologies Inc., Shanghai, China) according to the manufacturer's instructions. Approximately 3×10^3 cells were seeded in 100 μ l per well RPMI 1640 or DMEM in a 96-well plate. The panel of HeLa cells or C33A cells was treated with various concentrations (0, 30, 60, 90, 120, 150 pfu/ml) of Adv-Mfn2 or Adv-control, and the cells were cultured for different lengths of time (48 h and 60 h). Subsequently, 10 μ l of the CCK-8 solution was added to each well and incubated at 37 °C for another 2 h. The absorbance at 450 nm was measured on a spectrophotometric plate reader. Each group was repeated in three different wells.

Cell apoptosis analysis by flow cytometry

HeLa cells were seeded in 6-well plates for 24 h before being exposed to Adv-mfn2. After HeLa cells were treated with 90 or 120 pfu/ml of Adv-Mfn2 for 48 h and 60 h, the cells were harvested, washed with pre-chilled PBS twice and prepared in a single-cell suspension. Apoptosis was measured by a flow cytometer (BD Biosciences, USA) with FITC-conjugated Annexin V and propidium iodide (PI) double staining (Nanjing Biobox Biotech, China).

Mitochondrial membrane potential analysis by flow cytometry

HeLa cells were seeded in a 6-well tissue culture plate. Adv-control and Adv-mfn2 were added into the medium after 24 h. After the HeLa cells were treated for 60 h with 90 or 120 pfu/ml of Adv-Mfn2, the alteration of the mitochondrial membrane potential was measured using the Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, Nanjing, China). After incubation with JC-1 fluorescence dye for 30 mins, the cells were washed and resuspended with JC-1 Assay Buffer. Flow cytometry analysis was performed using a flow cytometer (BD Biosciences, USA).

Xenografted cervical carcinoma mouse model

BALB/c nude mice (4-5 weeks old) were obtained from the Hubei Provincial Centre for Disease Control and Prevention (HBCDC, China). We collected 6×10^5 human cervical carcinoma HeLa cells and injected them

subcutaneously in the right armpit of nude mice. When the tumours were visible, the mice were divided into 2 groups consisting of 8 tumour-bearing mice per group for a 9-day treatment with Adv-Mfn2 and Adv-control, respectively. Adenovirus was injected into the tumour tissue. Every three days, a 100- μ l solution of Adv-control (5×10^8 pfu/ml) or Ad-Mfn2 (5×10^8 pfu/ml) was injected into the tumours per mouse, which were determined from our preliminary experiment. All animal studies were carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" and approved by the Hubei Provincial Centre for Disease Control and Prevention (HBCDC, China).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from the HeLa cells or tumour issues was extracted using TRIzol® Reagent (Invitrogen, USA). Reverse transcription and qRT-PCR were performed as described previously [27]. Amplification and detection of specific products was performed with ABI step one plus (PE Applied Biosystems). RevertAid Reverse Transcriptase (Thermo scientific, EP0442) and qPCR Master Mix (Fermentas, K0221) were used to measure the mfn2 levels. GAPDH was used as an internal control for mfn2 RNA. Data were analysed using the $2^{-\Delta\Delta Ct}$ method. The following sequence-specific primers were used to amplify the gene products: mfn2, F:5'-ATCTGTGCCAGCAAGTTGACA-3', R:5'-AAGTGAATCCAGAGCCTCGAC-3', and GAPDH, F:5'-GACAAAATGGTGAAGGTCGGT-3', R:5'-GAGGTCAATGAAGGGGTCG-3'.

Western blotting

Whole cell proteins were extracted with a RIPA lysis buffer *in vitro* experiment, while the tumour tissues in the xenograft experiment were homogenized with ice-cold lysis buffer for total protein extraction. Protein concentrations were determined using the BCA protein assay kit (Pierce, 23227) according to the manufacturer's instructions. The proteins were fractionated on a 12% and 15% SDS-polyacrylamide gel and electroblotted onto Immobilon-P PVDF transfer membranes (Millipore, IPVH08130), as recently reported [28]. The blots were incubated with Mfn-2, anti-p53, cleaved caspase-3, cleaved caspase-9, cyto c, Bax, Bcl-2, Bid and p-Bad antibodies. GAPDH and β -actin were used as loading controls. The positive signals were visualized by Odyssey® Two-Color Infrared Imaging System (Li-Cor, USA).

Detection of xenografted tumour growth and TUNEL assay

The tumour volume was analysed at 0, 3, 6 and 9 days after starting the protein treatment, and the volume of the tumours was measured by the following formula: volume = $0.52 \times \text{length} \times \text{width}^2$ [28]. After a 9-day treatment, the tumour tissues were obtained by sacrificing the mice, and the tumours were dissected and fixed in 4% paraformaldehyde. Paraffin sections were prepared for TUNEL assays (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling assay) by means of the *In situ* Cell Death Detection Kit, POD (Roche, 11684817910). Image-Pro Plus was used to quantify each picture.

Immunohistochemistry

After a 9-day treatment, the mice were sacrificed, and the tumours were fixed with 4% paraformaldehyde. Paraffin sections were prepared for immunohistochemistry. The presence of cleaved caspase-3 was analysed by means of corresponding antibodies that were recently reported in our paper [28]. Image-Pro Plus was used to quantify each picture.

Statistical analysis

Statistical analyses were carried out in GraphPad Prism. The Student's t-test was used to determine the statistical significance of the data. A *p* value less than 0.05 (*) or less than 0.01 (**) was considered to be significant. Data presented in the figures represent the mean $\pm$ SD.

Results

Effects of Mfn2 on human cervical carcinoma HeLa cells *in vitro*

To examine the effects of Mfn2 on the viability of human cervical carcinoma HeLa cells, a panel of HeLa cells was treated with various concentrations (0 to 150 pfu/ml) of Adv-Mfn2. Meanwhile, effects of Mfn2 on C33A cells, which were HPV negative cervical cancer cells, were

investigated. As shown in Fig. 1A and 1B, the exposure of HeLa cells or C33A cells to Adv-Mfn2 resulted in a decrease in cell proliferation. Western blot analysis confirmed that Mfn2 expression increased in HeLa cells infected with Adv-Mfn2 compared with the Adv-control group and the blank control group (Fig. 1C). The data suggested that Mfn2 overexpression could inhibit HeLa cell proliferation in a concentration- and time-dependent manner.

Mfn2 induced apoptosis of HeLa cells

To examine the possible mechanism of Mfn2 inhibition of cell viability, apoptosis was investigated after Adv-Mfn2 treatment of HeLa cells. We speculated that Mfn2 could further lead to programmed cell death in HeLa cells due to the effects of Adv-Mfn2 on proliferation. To address this issue, we evaluated the apoptosis of HeLa cells treated with the indicated doses of Adv-Mfn2 for 48 h and 60 h using an Annexin V-FITC/PI staining assay. Phosphatidylserine

Fig. 1. The effects of Mfn2 on cell growth and apoptosis in HeLa cells. HeLa cells (A) and C33A cells (B) were exposed to different concentrations of Adv-Mfn2 (0, 30, 60, 90, 120, 150 pfu/ml) for 48 h and 60 h or Adv-control for 60 h and then the cell viability was measured using a CCK-8 assay. (C) Cell lysate was extracted from HeLa cells treated with DMSO, Adv-control, or Adv-Mfn2 (90 or 120 pfu/ml) for Mfn2 expression by Western blot analysis. GAPDH was used as a reference. (D) HeLa cells were incubated with Ad-Mfn2 for 48 h or 60 h. Cells were stained with Annexin V-FITC/PI after incubation with two concentrations (90 or 120 pfu/ml) of Adv-Mfn2 were measured by flow cytometry. (E) The percentage of cell apoptosis after HeLa cells were treated with the indicated concentrations of Adv-Mfn2. All results are expressed as the mean $\pm$ SD from three independent experiments (** $p < 0.01$ vs Adv-control group; ## $p < 0.01$ vs blank control group).

Fig. 2. Mitochondrial membrane potential decreased and the expressions of mitochondrial apoptosis-related proteins were regulated after being treated with Adv-Mfn2. (A) Mitochondria membrane potential decreased after HeLa cells were treated with Adv-Mfn2 for 60 h in a dose-dependent manner. (B) Dose-dependent changes in the mean JC-1 fluorescence after HeLa cells were treated with Adv-Mfn2 for 60 h (mean $\pm$ SD from three independent experiments, ** $p < 0.01$). (C) Adv-Mfn2 increased the release of cytochrome c into the cytosol by Western blot. Beta-actin was used as reference. (D) The expression of p53 protein in the cervical tumour HeLa cells was detected by Western blot. GAPDH was used as a reference. (E) Expressions of the mitochondrial apoptosis-related proteins, such as cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 after HeLa cells were treated with Adv-Mfn2 for 60 h. Beta-actin was used as reference.

expressed on the cell surface, which is an event identified in apoptosis, can be detected using Annexin V as a probe. As shown in Fig. 1D and 1E, HeLa cells displayed apparent apoptosis after treatment with Adv-Mfn2 in a dose-dependent manner. Taken together, these data revealed that Mfn2 could induce apoptosis in HeLa cells.

Mfn2 mediated mitochondrial membrane potential and cytochrome c release

Apoptosis is often accompanied by a decrease in the mitochondrial membrane potential (MMP) due to the disruption of the mitochondrial integrity, which is an early event and standard evidence in mitochondrial pathway apoptosis.

The cyanine dye JC-1 could form J-aggregates that are spectrally distinguishable from the dye monomers at high concentrations in the energized mitochondria of cells exposed to near-micromolar external concentrations of dye, which makes the JC-1 assay widely used for the measurement of MMP [29]. JC-1, as a monomer at low membrane potentials, and JC-1, as J-aggregates at high membrane potentials, could be measured by flow cytometry to determine the MMP. Therefore, we examined the changes of the MMP in HeLa cells after treatment with Adv-Mfn2 or Adv-control for 60 h. Interestingly, we found that the mitochondrial membrane potential was clearly decreased in a dose-dependent manner (Fig. 2A and 2B). These data

suggest that Mfn2 was able to affect the mitochondrial membrane potential of HeLa cells. Mitochondrial membrane depolarization is an early indicator of apoptosis, which causes the release of cytochrome c from the mitochondria into the cytosol and initiates a signalling cascade that executes cell death by apoptosis [30]. As evidenced by Western blot data (Fig. 2C), Adv-Mfn2 increased the release of cytochrome c from the mitochondria into cytosol.

Mfn2 induced activation of caspase-3 and caspase-9 in HeLa cells

Apoptosis is mediated by the activation of caspase cascades, which initiate and execute apoptosis. Earlier, it was reported that the activation of caspase-9 was linked with the release of cytochrome c from the mitochondria into the cytosol and the induction of apoptotic signalling pathways [31]. Western blot results showed that the expression of p53 protein was obviously upregulated compared with the Adv-control group (Fig. 2D). To determine the role of caspase-3 and caspase-9 in the Mfn2-induced mitochondrial membrane depolarization in HeLa cells, we next examined the levels of activated caspase-3 and caspase-9 using Western blot. As shown in Fig. 2E, the higher activity of cleaved caspase-3 and cleaved caspase-9 in HeLa cells was associated with higher Adv-Mfn2 concentration. The data suggested that the mitochondrial apoptosis pathway induced by Mfn2 in HeLa cells was related with the activation of caspase-3 and caspase-9.

Mfn2 regulated the expression of Bcl-2 and Bax in the mitochondria apoptosis signalling pathway

The Bcl-2 family, which is important in the mitochondria apoptosis cascade, consists of pro-apoptotic and anti-apoptotic proteins. To further explore the molecular mechanism

Fig. 3. Mfn2 is taken up into the tumours of the *in vivo* cervical carcinoma model. (A) The xenografted cervical carcinoma model in the right armpit of nude mice. (B) The expression of mfn2 RNAs in the tumours of the mouse model were detected by qRT-PCR quantitation. Data are represented as the mean $\pm$ SD (** p <0.01). (C) and (D) The expression of Mfn2 proteins in the tumours of the mouse model were detected by Western blot (C) and quantitation (D). Data are represented as the mean $\pm$ SD (** p <0.01).

behind the mitochondria-dependent apoptosis induced by Mfn2, we examined the expression of Bcl-2 family proteins, including the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. As seen in Fig. 2E, the overexpression of Mfn2 could upregulate the Bax protein level and downregulate the level of Bcl-2 compared to the control group, which suggested that the decrease of the Bcl-2/Bax ratio was involved in apoptosis induced by Mfn2.

Overexpressed Mfn2 in tumour tissues of the cervical carcinoma mouse model

The human cervical carcinoma HeLa cells were injected subcutaneously on the right armpit of female BALB/c nude mouse. After 5 days, the tumour tissues were found on the right armpit of the mice (Fig. 3A), and the volumes were over $3 \times 3 \text{ mm}^2$. The Adv-Mfn2 and Adv-control were injected into the tumours. The tumour tissues were obtained 72 h after injection with Adv-mfn2 or Adv-control, and the Mfn2 mRNAs were detected by qRT-PCR (Fig. 3B), while the proteins were examined by Western blot (Fig. 3C and 3D). We found that the RNAs and proteins of mfn2 were over expressed in tumour tissue after 72 h of treatment with Adv-Mfn2.

Mfn2 inhibited the growth of cervical carcinoma in vivo

To verify the effects of Mfn2 against cervical carcinoma *in vivo*, the therapeutic effects of Mfn2 on tumour tissues in the xenografted cervical carcinoma mouse model for a 9-day-treatment were assessed. HeLa cells were injected subcutaneously, and the animals were randomly divided into two groups of 8 tumour-bearing mice each. The bigger tumour-bearing mice were treated with Adv-Mfn2, and the other group was treated with Adv-control as described in the Materials and Methods section. At days 0, 3, 6 and 9 during the

Fig. 4. Mfn2 inhibited tumour growth in an *in vivo* cervical carcinoma model. (A) Determination of the tumour volume in mice treated with Adv-mfn2 or Adv-control for 0, 3, 6 and 9 days. Data are represented as the mean $\pm$ SD (**p<0.01). (B) Western blot showed p53 protein levels in cervical tumour cells. The cervical tumour cells were prepared from total tumour tissue lysates and analysed by Western blot. GAPDH was used as reference. (C) TUNEL assay of the tumour sections from animals treated with either Adv-control or Adv-Mfn2. Cells that underwent apoptosis by Adv-mfn2 treatment were recognized by brown cell staining. The nuclei of the tumour cells appeared blue. Magnification: 200 $\times$. (D) Quantification of TUNEL positive cells of the tumour sections from animals treated with either Adv-control or Adv-Mfn2 (**p<0.01 vs Adv-control group).

Fig. 5. Mfn2 triggered cervical tumour cell apoptosis via the mitochondrial pathway. (A) Western blot showed cleaved caspase-3 protein levels in the cervical tumours treated with Adv-mfn2 after 9-day-treatment. Total tumour tissue lysates were prepared and analysed by Western blot for cleaved caspase-3 protein levels. GAPDH was used as reference. (B) The picture of the immunohistochemical staining represented the expression of cleaved caspase-3 protein levels between the Adv-Mfn2 group and Adv-control group as detected by anti-cleaved caspase-3 staining. Magnification: 400 $\times$. (C) Quantification of the expression of cleaved caspase-3 protein levels between the Adv-Mfn2 group and Adv-control group (** $p < 0.01$ vs Adv-control group). Western blot (D) and quantitation (E) showed Bax, Bcl2, Bid and p-Bad protein levels in the cervicultumour cells. The cervicultumour cells were prepared from total tumour tissue lysates, and analysed by Western blot. GAPDH was used as reference.

treatment, the volume of the tumours was analysed (Fig. 4A). After treatment, the volume of the xenografted cervical carcinomas treated with Adv-mfn2 declined gradually, whereas the volume of the tumours treated with Adv-control increased. At day 9, the tumour tissues were obtained, and the expression of the p53 protein was obviously upregulated compared with the Adv-control group analysed by Western blot (Fig. 4B).

After the treatment, the mice were sacrificed, and the tumours were analysed by means of a TUNEL assay (Fig. 4C, 4D). The Adv-Mfn2 group underwent massive apoptosis, as indicated by the observed brown colouring in the analysed histological sections. In contrast, the tumours treated with Adv-control had no significant signs.

Mfn2 induced HeLa tumour tissues to apoptosis in mouse model

To confirm the apoptosis induction in HeLa tumour tissues by Adv-Mfn2, the active executioner caspase-3 was analysed by Western blot and immunohistochemical staining. As shown in Fig. 5A, Western blot analysis showed that caspase-3 was activated in tumours of the Adv-Mfn2 group compared with the Adv-control group after 9 days of treatment. Subsequently, sections of all the treated tumours were prepared for the detection of active executioner caspase-3 by staining the tumour sections with anti-cleaved caspase-3 antibodies directed against the cleaved caspase-3 present in the cervical tumour tissues. Fig. 5B and 5C showed that the cleavage caspase-3 proteins appeared in large parts of the Adv-Mfn2-treated tumours compared with the control group. These data demonstrated that Mfn2 could reduce the size of xenografted cervical carcinoma *in vivo* and induced apoptosis in the cervical tumour.

Mfn2 induced cervical carcinoma to apoptosis via the mitochondrial pathway

The Bcl-2 family, which is known to play a relevant role in the development of cervical carcinoma, comprises two groups of proteins with distinct functional roles in the intrinsic pathway of apoptosis in that they harbour anti-apoptotic or pro-apoptotic activity [29]. The Bcl-2 protein is an anti-apoptotic member that is discovered first in the Bcl-2 protein family and associates with drug resistance in human lymphomas, while the Bax, Bad and Bid proteins are pro-apoptotic members [32, 33].

We examined whether Mfn2-mediated activation of caspase-3 was affected by the cellular level of the Bcl-2 family proteins. The cervical tumour tissues injected with Adv-Mfn2 or Adv-control were prepared for testing. The results represented in Fig. 5D and 5E demonstrated that the overexpression of Mfn2 could not only increase the levels of Bax and Bid in cervical tumour tissues but also decrease the phosphorylation of Bad and the expression of Bcl-2.

Therefore, we concluded that Mfn2 apparently induced apoptosis by activating caspase-3 through the mitochondrial apoptosis pathway. Nevertheless, the fact that the mitochondrial outer-membrane protein Mfn-2 highly increased in cervical tumour cells, demonstrated that apoptosis occurred via the mitochondrial apoptosis pathway.

Discussion

In the current study, we confirmed the cell apoptotic effect of Mfn2 on cervical carcinoma HeLa cells *in vitro* and *in vivo*. Data from CCK-8 and flow cytometry showed that Mfn2 could inhibit proliferation and induce apoptosis in a dose- and time-dependent manner in HeLa cells and C33A cells. The JC-1 Kit assay results revealed that the membrane potential of the mitochondria decreased in a dose-dependent manner in HeLa cells after Adv-Mfn2 treatment. The Western blot data confirmed higher cytosolic amounts of cytochrome c with increasing doses of Adv-Mfn2, which signified the onset of the intrinsic apoptotic pathway. To further validate apoptotic control by Mfn2, expression levels of important apoptotic proteins were analysed by Western blot. Levels of cleaved caspase-3 and cleaved caspase-9 increased in HeLa cells with Adv-Mfn2 treatment. We also found significant increases in Bax levels and decreased Bcl-2 levels with Adv-Mfn2 treatment. These are consistent with findings from the data, which confirmed that Mfn2 could induce apoptosis in the mitochondrial pathway *in vivo*. We examined the cervical tumour tissues from the nude mouse model of HeLa cells in the Ad-mfn2 group, of which p53 expression is upregulated. In HeLa cells, Mfn2 may inhibit HPV E6, which is combined with p53 [34], and inhibits the proliferation of HeLa cells. Meanwhile, Mfn2 is a novel direct target of p53 [35], and there may be positive feedback between Mfn2 with p53 [36]. Mfn2 may inhibit C33A cell proliferation through a positive feedback relationship with Mfn2 and p53. We also found that Mfn2 could inhibit cervical tumour size after injection with Ad-mfn2 and induce cervical tumour cells into apoptosis. To discuss the molecular mechanism of Mfn2 in triggering cervical tumour apoptosis, we analysed the expression of Bcl-2 family proteins. The results showed that Mfn2 upregulated pro-apoptotic proteins of Bcl-2 family members as Bax in the mitochondrial apoptosis pathway and downregulated anti-apoptotic proteins such as Bcl-2. These preclinical studies all revealed the therapeutic potential of the Mfn2 protein for anti-cancer treatment.

Mitochondria, the intra-cellular unique DNA-containing organelle whose most common function is the production of adenosine triphosphate (ATP) in the presence of oxygen, is also a biologically important source and target for reactive species. Thus, mitochondria play a key role in cancer and its progression. Except for regulating mitochondrial fusion, the mitochondrial outer membrane protein Mfn2 has been proved pivotal in maintaining mitochondrial morphology and homeostasis. In addition, studies have shown that Mfn2 regulates cell proliferation and apoptosis associated with mitochondria [13]. The cytosolic release of cytochrome c, which inflicts an irrevocable activation of caspase and subsequent cell death, which is due to the damaged mitochondrial membrane [37, 38]. Although the

mechanisms regulating mitochondrial permeability and the release of cytochrome c during apoptosis are not fully understood, Bcl-xL, Bcl-2, and Bax may influence the voltage-dependent anion channels, which may play a role in regulating cytochrome c release. The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax, and Bim may reside in the cytosol but translocate to mitochondria following death signalling, where they promote the release of cytochrome c. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-xL.

Bcl-2 is a major anti-apoptotic Bcl-2 family member that is directly involved in the suppression of apoptosis at the decision stage [39]. Bcl-2 can inhibit apoptosis induced by p53 [40]. On the other hand, Bax is a major pro-apoptotic Bcl-2 family member that has been identified as a promising prognostic indicator in patients with lung cancer [41], and a molecule required for cell death that is extensively expressed in both small cell (SCLC) and non-small cell lung cancer (NSCLC) cells [42-44], suggesting that it could potentially also offer a therapeutic target in cancer patients. The target, which influences the expression and function of Bax and Bcl-2, could affect cancer and its progression. Mimicking the BH3 domain to induce apoptosis has recently been used as a strategy for the development of Bcl-2 inhibitors as anticancer drugs [45-48]. The BH3 mimetic agents function as competitive inhibitors by binding to the hydrophobic cleft of Bcl-2/Bcl-XL [45, 48]. Four Bcl-2 inhibitors have already been tested in human clinical trials but showed limited clinical efficacy [42, 43, 45, 46]. The multi-domain pro-apoptotic Bax provides the required gateway to apoptotic cell death [48, 49]. Many times, it was focused on either Bax upregulation or Bcl-2 inhibition. In mitochondria, Mfn2 upregulated pro-apoptotic Bax and downregulated anti-apoptotic Bcl-2 at the same time, inducing HeLa cells into apoptosis via the mitochondrial pathway.

We found that Mfn2 could activate Bax and inhibit Bcl-2 expression in the mitochondrial pathway, which provided a strong rationale that Mfn2 combined anti-apoptotic protein inhibition and pro-apoptotic protein activation, which should have superior therapeutic benefits, and effectively triggered tumour cells into apoptosis. Based on our data, we propose that Mfn2, which co-targets pro-apoptotic and anti-apoptotic proteins in the mitochondrial pathway, offers a more effective strategy for cervical carcinoma treatment.

Conclusion

Our studies demonstrated that Mfn2 can induce cervical carcinoma HeLa cell apoptosis via the mitochondrial pathway. It activated the pro-apoptotic processes in the mitochondrial apoptosis pathway and inhibited anti-apoptotic protein expression in the mitochondrial pathway. All these features illustrate that Mfn2 may be a promising starting point for the future clinical application of efficient anticancer treatment targets in the cervical carcinoma therapy.

Acknowledgements

Our animal studies were carried out according to the Guide for the Care and Use of Laboratory Animals of the People's Republic of China in strict compliance. All efforts were made to minimize suffering and all procedures were performed under ethyl ether anaesthesia. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Chinese Centre for Diseases Control and Prevention.

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