Original Article

MDA-7/IL-24 inhibits cell survival by inducing apoptosis in nasopharyngeal carcinoma

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Received September 3, 2014; Accepted October 23, 2014; Epub November 15, 2014; Published November 30, 2014

Abstract: Aims: Nasopharyngeal carcinoma (NPC) is the most common primary malignancy of the nasopharynx. Due to its local recurrence and distant metastasis, conventional therapy is usually ineffective. MDA-7/IL-24 (melanoma differentiation associated gene 7), a member of the IL10 family of cytokines, inhibits growth of various human cancer cells, but the underlying mechanism is largely unknown. There is no report of mda-7 in nasopharyngeal carcinoma. We aimed to investigate the role of MDA-7/IL-24 in NPC. Methods: Immune defective adenoviral vector carrying the gene was produced, infected NPC CNE cells and observed its growth, cell proliferation, apoptosis and the effect of combination with chemotherapy. Results: The results showed that (1) MDA-7/IL-24 inhibited NPC CNE cell growth and survival; (2) mda-7 induced cell apoptosis and death; (3) MDA-7/IL-24 in collaboration with chemotherapy induced cell apoptosis significantly; (4) MDA-7/IL-24 induced cell apoptosis by down-regulation of anti-apoptosis molecules such as Bcl-2 and Bcl-xl and up-regulation of caspase 3. Conclusion: The results suggested that MDA-7/IL-24 had obvious therapeutic effect in NPC cells. It is verified that adenovirus mediated MDA-7/IL-24 represents a potentially important new approach to NPC therapy.

Keywords: MDA-7/IL-24, nasopharyngeal carcinoma, cell survival

Introduction

Nasopharyngeal carcinoma (NPC) is the most common primary malignancy of the nasopharynx. In the past, radiotherapy was the therapy of choice, but this modality is limited due to recurrence and metastasis. It is very important to find alternative methods such as biological therapy to treat NPC.

MDA-7, which is classified as a member of the interleukin (IL)-10 gene family and designated as MDA-7/IL-24, was cloned from HO-1 cells induced by IFN-β and MEZ [1]. The human MDA-7/IL-24 gene is located in 1q32-33 and belongs to the member of the IL-10 family and is named as IL-24. Multiple studies have demonstrated that expression of MDA-7/IL-24 in a wide variety of tumor cell types, but not in corresponding equivalent non-transformed cells, causes growth arrest and rapid cell death [2-5].

In addition, MDA-7/IL-24 has been noted to radiosensitization in tumor cells, partially due to the generation of reactive oxygen species (ROS) and ceramide that cause endoplasmic reticulum stress and suppress protein translation. Phase I clinical trial data has shown that a recombinant adenovirus expressing MDA-7/IL-24 (Ad.mda-7 (INGN-241)) is safe and has measurable tumoricidal effects in over 40% of patients, strongly arguing that MDA-7/IL-24 could be of significant therapeutic value [6-10]. MDA/IL-24 has two major targets in cells: the IL-20/IL-22 receptor complexes and the HSP70 family chaperone GRP78/BiP. MDA-7/IL-24 binding to its cognate receptors activates STAT family transcription factors and activation of these factors can promote differentiation and proliferation in a cell type-dependent manner [11, 12]. MDA-7/IL-24 binds to GRP78/BiP; it is possible that entry of bacterial synthesized GST-MDA-7 into tumor cells is mediated by binding to cell surface GRP78/BiP. The majority of GRP78/BiP is present in the endoplasmic reticulum and is bound to PKR-like endoplasmic reticulum kinase (PERK); the chaperone inhibits...
PERK kinase activity. MDA-7/IL-24 disrupts the association of GRP78/BiP with PERK, permitting PERK to phosphorylate eIF2α; phospho-eIF2α suppresses the translation of the majority of cellular proteins, resulting in rapid loss of protective proteins that have short half lives such as MCL-1 and BCL-XL, and via ATF4 promotes the transcription of a specific subset of genes, e.g. GADD34, that promote apoptosis. PERK signaling promotes increased LASS6 (ceramide synthase 6) levels that promote increased Ca²⁺ mobilization leading to elevated ROS levels. Increased ceramide/Ca²⁺/ROS activate JNK and p38 signaling that promotes activation of the toxic BH3 domain proteins BAX and BAK [13-19].

It was reported that MDA-7 can inhibit tumor cell growth and induce apoptosis in malignant melanoma, breast cancer, liver cancer, prostate cancer, and ovarian cancer except for small lung cancer and pancreatic cancer [20-24]. But there are no reports in NPC. Therefore, in our research, we used NPC cell line CNE transduced with mda-7 adenovirus to observe tumor cell survival, proliferation and apoptosis. To further investigate the molecular mechanism in the apoptosis of MDA-7, the apoptosis and anti-apoptosis molecules were detected. We also combined mda-7/IL-24 with chemotherapy to induce cancer cell apoptosis to verify the effect of mda-7/IL-24 with chemotherapy.

Materials and methods

Cell lines and cell culture

CNE cells were purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China, and maintained in the RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO₂ at 37°C. 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C.

Adenoviruses

Adenoviral vectors pAd-mda7 and pAd-GFP carrying the MDA-7/IL-24 and GFP gene, respectively, were digested with PacI, and the DNA was recovered for transfection of 293T cells. After 2 to 3 weeks, GFP was observed and the medium was collected including the cells and centrifuged at 1000 rpm for 8-10 min. The supernatant containing the viruses was saved and the adenoviruses carrying mda-7 and GFP were named as AD-GFP and AD-mda-7, respectively. The viruses were titrated and cells were infected by the AD-GFP and AD-mda-7 adenoviruses at a multiplicity of infection (MOI) of 30.

Biological behavior such as cell growth and apoptosis was analyzed after infection at the indicated time points.

The MTT and TUNEL assays

Cellular proliferation was evaluated using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyethylazolium bromide colorimetric assays as instructed by the manufacturer (Sigma, USA). Briefly, cells were seeded at 1×10⁴ cells/well in 96-well plates. Absorbance was read at 570 nm. Each experiment was carried out in triplicate and at least three times independently. Cell apoptosis was examined using the TdT-mediated dUTP nick end-labeling (TUNEL) assays as instructed by the manufacturer (Beyotime, China).

Propidium iodide staining

The cells were washed with PBS for two times, and propidium iodide was added (Beyotime, China) at 4°C for 10 min, and washed with PBS for three times, and the cells were then observed.

Apoptosis assays

Cells were harvested and washed once with phosphate-buffered saline (PBS), trypsinized, and washed again in PBS with 2% FBS and resuspended in binding buffer containing 10 mM HEPES (pH 7.4); 2.5 mM CaCl₂, and 140 mM NaCl, and stained with annexin V-PE and propidium iodide (30 g/mL) according to the manufacturer’s protocol (Southern Biotech, Birmingham, AL). Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using Cell quest software, and the ModFit program (Verity Software House Inc., Topsham, ME) was used to analyze apoptosis. Each experiment was conducted in triplicate and at least three times independently.

Apoptosis assay

Apoptosis was measured using an Annexin V/propidium iodide (PI) apoptosis detection kit (Bender MedSystem, Vienna, Austria). Briefly,
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Figure 1. MDA-7/IL-24 inhibits CNE cell proliferation. CNE cells were infected with AD-GFP or AD-mda-7 at an MOI of 30. A. CNE cells were infected with AD-GFP and AD-mda-7, respectively. B. Cell growth curve was plotted using cell counts at the indicated time points. C. Cell proliferation detection by the MTT assays. Cells with AD-mda-7 or AD-GFP infection were seeded in 96-well plates at the indicated time points.

Cells cultured in 6-cm dishes were trypsinized, washed, stained with PI-conjugated anti-Annexin V antibody under darkness for 15 min at room temperature, and then analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, Mountain View, CA, USA).

RT-PCR assays

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using a Reverse Transcriptase kit (Invitrogen) following the manufacturer’s instructions. The following primers were used for PCR: GAPDH, 5'-GTCTCCTCTGA-CTCAACAGCG-3' (sense) and 5'-ACCACCCTG-TTGCTGTAGC-3' (antisense); Bcl-xl, 5'-GCCACCTGAATGACCACC-3'(sense) and 5'-AA-CCAGCGTGAAGCGTTCTC-3'(antisense); Bcl-2, 5'-GTGTCTCTCTGGTGTTATCAG-3' (sense) and 5'-GCCAGGAAATCAAACAGGCC-3' (antisense); MDA-7 (IL-24), 5'-CTTCTCTGGAGTTGTTATCAGA-3' (sense) and 5'-GGCAGTGTGAGTTATCAGG-3' (antisense). PCR was performed in a 100 μL reaction containing plasmid 1 μl; 10 x buffer, 10 μl; forward and reverse primers, 2 μl; dNTPs, 8 μl; Taq polymerase, 1 μl; dH2O, 78 μl and the PCR was run at 95°C, 2 min, 1 cycle; 95°C, 30 sec, 58°C, 30 sec, 72°C, 2 min, 30 cycles; 72°C, 6 min, 1 cycle. The PCR products were resolved by agarose electrophoresis.

Western blotting assays

Cellular lysates were prepared by homogenization in an ice-cold lysis buffer and proteins were quantified using the Bradford method and samples were resolved by SDS-PAGE. Immunoblotting was performed as previously described and the following antibodies were used: anti-Bcl-2 and anti-caspase3 antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized using ECL.

Statistical analysis

Each experiment was done at least three times, and Student’s t tests were performed to determine statistical significance for the assays of cell proliferation. Data were expressed as mean ± SE.
Figure 2. MDA-7/IL-24 promotes cell apoptosis. A. CNE cells were infected with AD-GFP or AD-mda-7, and labeled with TUNEL to observe cell apoptosis. B. CNE cells were infected with AD-GFP or AD-mda-7, and stained with propidium iodide to observe cell death. C. CNE cells were infected with AD-GFP or AD-mda-7, and labeled with Annexin V to observe cell morphology.
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Figure 3. MDA-7/IL-24 promotes cell death when combined with Ara-C. A. CNE cells were treated with Ara-C with or without AD-mda-7, and then the cells were collected for AnnexinV labeling for flow cytometry. B. CNE cells were treated with Ara-C with or without AD-mda-7, and then the cells were collected for propidium iodide staining for detection of cell death.

Results

MDA-7/IL-24 inhibits CNE cell proliferation

First, we constructed the adenovirus vectors AD-mda-7 and its control vector AD-GFP. Infection efficiency is shown in Figure 1A. Next, we investigated the effect of mda-7 on the proliferation of CNE cells by infecting these cells with AD-GFP and AD-mda-7, respectively. Cell counts showed that AD-mda-7 markedly inhibited the growth of CNE cells compared with cells infected with AD-GFP 4 days after treatment (Figure 1B). The MMT MTT assays indicated that AD-mda-7 caused a 72% reduction in the proliferation of infected cells 72 h after infection (Figure 1C).

MDA-7/IL-24 promotes cellular death

To elucidate whether MDA-7/IL-24 leads to cell apoptosis, we used FITC-labeled TUNEL to observe cell apoptosis. CNE cells were transfected with AD-GFP and AD-mda-7, respectively. As shown in Figure 2A, cells infected with AD-mda-7 showed significant apoptosis compared with cells infected with AD-GFP and PBS controls. Meanwhile, propidium iodide staining showed that AD-mda-7 caused significant cellular death compared with cells infected with AD-GFP and PBS controls (Figure 2B). Flow cytometry of annexin V stained cells further showed that AD-mda-7 caused a significant increase in cellular death compared with the other two groups.

MDA-7/IL-24 combined with Ara-C promotes cell death

Ara-C is used in the clinic for anti-tumor drug, but there is no good effect on NPC. We want to know if there was good therapeutic effect when the drug was combined with mda-7. CNE cells were treated with Ara-C with or without AD-mda-7.
7. Annexin V assays showed that Ara-C alone did not cause significant cellular death, but when it was combined with AD-mda-7, a significant increase in cell death was observed (Figure 3A). Propidium iodide staining showed a significantly greater number of dead cells (>90%) treated with Ara-C and mda-7 compared to cells treated with Ara-C only (Figure 3B).

**MDA-7/IL-24 induces CNE cell death by the caspase-3 pathway and by down-regulation of anti-apoptosis protein**

Based on the above data, to further investigate the mechanism of cell death induced by mda-7, we infected CNE cells with AD-GFP or AD-mda-7. The results showed that Bcl-2 and Bcl-xl mRNA transcript levels were decreased in cells infected with AD-mda-7 (Figure 4A). The result suggested that mda-7 may induce NPC cell death through down-regulation of Bcl-2 and Bcl-xl. The result of Western blotting further indicated that Bcl-2 protein was decreased and caspase3 increased significantly in cells infected AD-mda-7 (Figure 4B). These data suggested that mda-7 promoted cell death by the caspase-3 pathway and by down-regulation of anti-apoptosis protein.

**Discussion**

NPC is a common carcinoma in the head and neck. It is resistant to regular therapeutics because of its recurrence and metastasis. It is very important to find alternative methods such as biological therapies. It was reported that MDA-7/IL-24 can inhibit tumor cell growth and induce apoptosis in malignant melanoma, breast cancer, liver cancer, prostate cancer, and ovarian cancer except for small lung cancer and pancreatic cancer. But there are no reports in NPC.

Previous report showed that MDA-7/IL-24 can inhibit breast cancer and lung cancer growth or induce apoptosis through Wnt/β-catenin, PI-3K or p38-MAPK signaling pathways [25-27]. In our research, we constructed adenovirus mediated MDA-7/IL-24 and infected NPC cells. It was found that MDA-7/IL-24 could inhibit CNE cell proliferation and growth by inducing cell apoptosis and death. When the cells were infected with MDA-7/IL-24, they showed significant cellular death.

Some reports indicated that the MDA-7/IL-24 gene might induce tumor cell apoptosis through various signaling pathways, not dependent on the activity of p53, PRB, and p21. It also inhibits tumor angiogenesis [28, 29] and cell invasion and migration [30, 31], which made it a good therapeutic gene clinically. MDA-7/IL-24 is a secreted cytokine, which makes it have the “by-stander” function. In a phase I Ad-mda7 (INGN 241) clinical trial study, it was found that MDA-7/IL-24 was expressed in 30% 100% of tumors which showed and induced apoptosis in 70% of tumors [32, 33]. Our findings showed that mda-7/IL-24 induced cell death through down-regulation of anti-apoptosis protein such as Bcl-2, Bcl-xl and up-regulation of caspase 3. Further studies are needed to detect other
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apoptosis associated proteins or pathways in CNE cells infected with AD-GFP-mda-7. We tried to observe CNE cell metastasis, but due to significant cell death, the results would be affected. In the future, we want to construct stable cell lines to study other biological behaviors of CNE cells such as invasion, migration and etc. We also found that CNE cells infected with mda-7 adenovirus in combination of Ara-C showed significant cell death compared with cells treated with Ara-C. These suggested that it was very difficult to use only one drug to treat cancer.

In conclusion, MDA-7/IL-24 can inhibit cancer cell proliferation, and induce apoptosis and death. CNE cells not sensitive to Ara-C therapy are sensitized to Ara-C combined with MDA-7/IL-24. MDA-7/IL-24 is a potential target gene in tumor therapy.

Acknowledgements

The study was approved by the Shanghai Committee of Science and Technology key basic research projects.

Disclosure of conflict of interest

None.

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