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Original Articles

Curcumin promotes apoptosis by activating the p53-miR-192-5p/215-XIAP pathway in non-small cell lung cancer

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ABSTRACT

Curcumin has attracted increasing interest as an anti-cancer drug for decades. The mechanisms of action involve multiple cancer-related signaling pathways. Recent studies highlighted curcumin has epigenetic regulatory effects on miRNA in cancers. In the present study, we demonstrated the proapoptotic effects of curcumin *in vitro* and *in vivo*. miRNA microarray and qPCR indicated that miR-192-5p and miR-215 were the most responsive miRNAs upon curcumin treatment in H460 and A427 cells. Functional studies showed miR-192-5p/215 were putative tumor suppressors in non-small cell lung cancer. Curcumin also promoted miR-192-5p/215 expressions in A549 cells (p53 wild type) but not in H1299 cells (p53-null). Conditional knockdown of p53 by tetracycline inducible expression system significantly abrogated curcumin-induced miR-192-5p/215 upregulation in the p53 wild-type H460, A427 and A549 cells. Conversely, ectopic expression of exogenous wild-type but not R273H mutant p53 in the p53-null H1299 cells enabled miR-192-5p/215 response to curcumin treatment. The proapoptotic effects of curcumin also depended on miR-192-5p/215 induction, and antagonizing miR-192-5p/215 expression attenuated curcumin-induced apoptosis in H460, A427 and A549 cells, but not in H1299 cells. Finally, X-linked inhibitor of apoptosis (XIAP) is proved to be a novel transcriptional target of miR-192-5p/215. Taken together, this study highlights that the proapoptotic effects of curcumin depend on miR-192-5p/215 induction and the p53-miR-192-5p/215-XIAP pathway is an important therapeutic target for non-small cell lung cancer.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide, and it is responsible for 80% of lung cancer cases. The outcome of standard chemotherapy remains disappointing [1]. It is therefore urgent to identify new strategies for NSCLC treatment. A large body of evidence suggests that phytochemicals have encouraging anti-cancer effects, one such agent is curcumin. Curcumin (diferuloylmethane), which is derived from the medical plant *Curcuma longa*, has attracted much attention over the past decade [2–4]. Curcumin has been shown to inhibit proliferation, and it potentiates chemotherapy in diverse cancers [5,6]. Epidemiological studies also indicate that a dietary curcumin regimen reduces the incidence of cancer, and the underlying mechanisms have been extensively studied [7–9]. Our previous study demonstrated that curcumin can manipulate hypoxia-inducible factor 1 alpha (HIF-1α) and overcome cisplatin resistance in lung adenocarcinoma A549 cells. Blocking caspase-3 with the pan-caspase inhibitor ZVAD-fmk attenuated apoptosis, indicating that curcumin induces apoptosis through a caspase-3-dependent manner [10]. Moreover, there is increasing lines of evidence suggesting that multiple cancer-related signaling pathways are responsible for the pharmacological effects of curcumin. These encouraging preclinical studies have prompted a great deal of completed and ongoing clinical trials for colon cancer, breast cancer, pancreas cancer, lymphoma and myeloma patients, and these trials have demonstrated that curcumin has anti-cancer activities with minimal adverse effects [11–14]. It is anticipated that

Abbreviations: NSCLC, non-small cell lung cancer; miR, microRNA; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PARP, poly ADP-ribose polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; DAB, diaminobenzidine; Tet system, tetracycline inducible expression system; Dox, doxycycline; shRNA, short hairpin RNA; 3'-UTR, 3'-untranslated region; qPCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; XIAP, X-linked inhibitor of apoptosis.

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curcumin or its analogs will eventually be clinically beneficial in cancer chemotherapy as a "standard drug" alone or in combination therapies.

miRNA play critical roles in multiple steps of carcinogenesis and serve as feasible therapeutic cancer targets [15]. For example, anti-oncogenic miR-26a is significantly downregulated in myc-induced murine liver cancer, and therapeutic delivery of miR-26a by the adeno-associated viruses has approached the attenuation of liver cancer development and progression [16]. Despite the success and high efficiency of miRNA-based therapies in many cancer models, it remains difficult to effectively and safely deliver miRNA *in vivo*. However, it is recognized that curcumin is an epigenetic agent serving as a new strategy for cancer treatment. Curcumin has initially been described for its epigenetic activity on miRNA in pancreatic cancer. Sun and colleagues reported that curcumin upregulates the expression of anti-oncogenic miR-22 while downregulating oncogenic miR-199a* in BxPC-3 pancreatic carcinoma cells [17]. Our previous studies indicated that curcumin provokes apoptosis by modulating miRNA expressions in lung cancer. Lung adenocarcinoma A549 cells treated with curcumin have an altered miRNA profiles including the downregulation of six miRNAs and upregulation of eight miRNAs. Among these miRNAs is oncogenic miR-186, which is related to A549 cells growth inhibition and apoptosis induction [18]. In addition, downregulation of miR-186 by curcumin in A549 cisplatin resistant counterpart cells is also involved in apoptotic cancer cell death [19]. Given that curcumin can regulate miRNA expressions, it would be a novel approach to "deliver" anti-oncogenic miRNAs or "block" oncogenic miRNAs for miRNA-based cancer therapies.

Moreover, the tumor suppressor p53 is frequently inactivated by mutations or deletions in cancer. p53 acts as an anti-oncogene through its manipulation of downstream targets, leading to cell cycle arrest, apoptosis and senescence. Recent studies have implied that miR-34 family members are direct transcriptional targets of p53. Upregulation of miR-34 leads to p53-like tumor suppressive effects, revealing an interplay between p53 and cancer-related miRNAs [20–22]. It is possible that other cancer-related miRNAs might be involved in the tumor-suppressive response upon p53 activation.

In this study, we report the global miRNA profiles elicited after curcumin treatment in H460 and A427 cells. miR-192-5p and miR-215 (miR-192-5p/215) are the most responsive to curcumin treatment, and they have been identified as putative anti-oncogenic miRNA in NSCLC. Importantly, curcumin upregulates miR-192-5p/215 through a p53-dependent mechanism. Conditional knockdown of wild-type p53 impairs the miR-192-5p/215 response in H460, A427 and A549 cells. In contrast, conditional expression of wild-type but not R273H mutant p53 elicits miR-192-5p/215 upregulation in response to curcumin treatment in p53-null H1299 cells. Finally, we report that X-linked inhibitor of apoptosis (XIAP) is a direct target of miR-192-5p/215. Targeting the p53-miR-192-5p/215-XIAP pathway by curcumin might be a novel therapeutic strategy for NSCLC.

Materials and methods

Cell culture

Human NSCLC H460, A427, A549 and H1299 cells were purchased from American Type Culture Collection (ATCC). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% FBS (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The histology and p53 genetic status of the cells are presented in Supplementary Table S1.

MTT assay

Cells were seeded at a density of 5×10^3 cells/well in 96-well culture plates. After attachment, cells were treated with various concentrations of curcumin (Sigma). Sterile MTT solution was added, and the cells were incubated for an additional 4 h. The supernatant in each well was carefully removed and formazan crystals were dissolved in 150 μl DMSO for 10 min with shaking. The absorbance of each well was then read at 570 nm using an enzyme-linked immunosorbent assay reader. The concentration at which curcumin produced growth 50% inhibition (IC_{50}) was calculated by a cell viability curve.

Annexin V-FITC/PI flow cytometry

After the indicated treatments, cells were harvested by trypsinization at 90% confluence. Apoptosis was measured with the Annexin V-FITC/PI kit (Millipore). Flow cytometry was performed using a Becton-Dickinson FACS-420 flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Tumor xenograft model

The *in vivo* cytotoxic effects of curcumin were assessed by subcutaneous injection of cancer cells into athymic nude mice (Laboratory Animal Center of Fourth Military Medical University). Animals were maintained under pathogen-free conditions at 25°C in a 12 h light/dark cycle. Approximately 2.0×10^6 cells suspended in 100 μl PBS were subcutaneously injected into the flanks of 8-week-old male BALB/c nude mice. When massive tumor formation was observed, the mice were then divided into two groups to receive vehicle as control and 300 mg/kg curcumin ($n = 6$ per group) for 15 days. Each mouse was intragastrically administered by gavage needle with 300 μl vehicle (0.5% carboxymethylcellulose sodium solution) or an equal volume of curcumin solution every day for 15 days. Tumor growth was monitored and calculated with the following formula: $V = 0.4 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). Tumor volumes were measured and recorded every 3 days. On day 16, the mice were sacrificed with CO_2, and the tumors were then removed and measured. All animal experiments were conducted in compliance with institutional guidelines and approved by the Animal Care and Use Committee of the Fourth Military Medical University.

Immunohistochemistry and TUNEL assay

Mice tumor xenograft specimens were fixed in 4% formalin for 24 h before being transferred to 70% ethanol. Tumor samples were subsequently paraffin-embedded, cut into 5 μm sections and baked on microscope slides. The slides were deparaffinized and hydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2. Antigen retrieval was performed with 10 mM citrate buffer. Slides were incubated with normal goat serum to block nonspecific binding, and they were incubated with various primary antibodies directed against Ki67 (Sigma), p53 (Sigma), p21 (Cell Signaling Technology) and XIAP (Cell Signaling Technology) overnight at 4 °C. The slides were then incubated with HRP-conjugated secondary antibodies for 60 min at room temperature and visualized with the Dako EnVision™ Detection kit (Dako). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Roche). Briefly, the slides were digested with proteinase K, blocked with 3% H_2O_2, and incubated with terminal deoxynucleotidyl transferase mixture for 60 min. The slides were thoroughly washed, incubated with streptavidin-HRP, and visualized by diaminobenzidine (DAB).

miRNA array

Total RNA was isolated with the TRIzol reagent (Invitrogen). The miRNA fraction was further purified using the mirVana™ miRNA Isolation Kit (Ambion). The isolated miRNA was then labeled with the Hy5™ and Hy3™ fluorescent labels using the Mercury Array Power Labeling Kit (Exelon) and hybridized to the miRCURY™ LNA miRNA Array (v. 10.0, Exiqon). Microarray images were acquired using the GenePix 4000B scanner (Axon Instruments) and analyzed with GenePix Pro 6.0 software (Axon Instruments), which performed median normalization.

Quantitative PCR and reverse transcription PCR

Total RNA was extracted with the TRIzol reagent according to the manufacturer's instructions (Invitrogen). First-strand cDNA was prepared from 1 μg of total RNA using the Thermoscript reverse transcription PCR system. The expression of mature miRNA was measured using stem-loop RT followed by qPCR analysis. All reagents for stem-loop RT were obtained from Qiagen. The relative amount of each miRNA was normalized to the amount of U6 snRNA. Primers sequences are listed in Supplementary Table S2. The fold change for each miRNA in curcumin-treated cells relative to the vehicle control (DMSO) was calculated using the $2^{-\triangle\triangle CT}$ method.

Reverse transcription PCR for p53 (forward: 5'-GTTTCCGTCTGGGCTTCT-3'; reverse: 5'-ACCTCAGGCGGCTCATAG-3'), p21 (forward: 5'-CCCGTGAGCGATGGAACT-3'; reverse: 5'-CGAGGCACAAGGGTACAAGA-3') and XIAP (forward: 5'-ACCGTGCGGTGCTTTAGTT-3'; reverse: 5'-TGCGTGGCACTATTTTCAAGATA-3') was performed according to the manufacturer's protocol (Takara). β-actin (forward: 5'-GACTACCTCATGAAGATC-3'; reverse: 5'-GATCCACATCTGCTGGAA-3') was used as an equal loading. Amplified PCR products were analyzed in a 1% agarose gel and visualized by ethidium bromide staining.