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Original Article

Curcumin inhibits the proliferation and invasion of human osteosarcoma cell line MG-63 by regulating miR-138

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Abstract: Objective: In this study, we screened the different human osteosarcoma cell line MG-63 miRNAs after the treatment of curcumin and explored the effects of curcumin on MG-63 cells and its mechanism. Methods: Affemitrix miRNA chip was used to detect the changes of miRNA expression profile in MG-63 cells before and after curcumin treatment, and screen different expression of miRNAs. The target gene of miRNA was analyzed by bioinformatics. The expression levels of miRNA-138 target genes Smad4, NFκB p65 and cyclin D3 were detected. MTT and Transwell Cell invasion assays were used to observe the effects of curcumin on MG-63 cells. Results: Curcumin could significantly inhibit the proliferation of MG-63 cells and the expression levels of miRNA-138 target genes Smad4, NFκB p65 and cyclin D3 in MG-63 cells ($P<0.05$); overexpression of hsa-miR-138 down-regulated the expression levels of Smad4, NFκB p65 and cyclin D3 compared with the treatment of curcumin, while inhibition of hsa-miR-138 up-regulated the expression levels of Smad4, NFκB p65 and cyclin D3. Conclusions: Curcumin could increase the expression of hsa-miR-138, hsa-miR-138 inhibited cell proliferation and invasive ability by inhibition of its target genes.

Keywords: Curcumin, miRNAs, miR-138, MG-63 cells, proliferation, invasion

Introduction

Curcumin is the main turmeric compounds in the spice of turmeric. It is a kind of lipid soluble phenolic pigment which is extracted from the roots of the plant Curcuma longa Linn. Curcumin has many pharmacological activities such as anti-inflammatory, anti-oxidation, lowering blood fat, anti-tumor and so on. It can inhibit the growth of a variety of tumor cells and induce apoptosis so as to exert its anti-tumor activity. Previous studies showed that curcumin induced the apoptosis of tumor cells in vivo and in vitro, such as lung cancer, colon cancer, breast cancer, pancreatic cancer, ovarian cancer and leukemia [1-3]. The drug development and molecular mechanism of anti-tumor researches based on curcumin had become a hot spot in the research of natural anti-tumor drugs.

Osteosarcoma is a malignant tumor originating from the tissues of the leaves, its incidence accounts for about 35% of the primary tumor, it was the most common primary malignant bone tumor in children and adolescents [4]. The prognosis of patients with osteosarcoma is very poor because of the high degree of malignancy and the ability of invasion and metastasis [5].

MicroRNAs (miRNAs) is a kind of non-coding RNA which is about 17-25 nucleotides in length, it participates in many life processes such as cellular differentiation, proliferation, apoptosis and tumor development. Although its sequence only accounted for about 1% of the human genome, it participates in the regulation of about 30% gene expression [6, 7]. Studies showed that miRNAs may be involved in the regulation of cell proliferation and differentiation by regulating the expression of target genes. So the expression profile of miRNAs could be a marker for early diagnosis and prognosis of cancer. Many abnormal expressions of miRNAs were found in osteosarcoma through miRNA expression profile recently [8-11].

Table 1. Primers used in real-time PCR

Gene	Accesion NO.	Primer (5'-3')
Smad4	NM_005359	F: CTCATGTGATCTATGCCCGTC
		R: AGGTGATACAACTCGTTCGTAGT
NFκB p65	NM_001145138	F: ATGTGGAGATCATTGAGCAGC
		R: CCTGGTCCTGTGTAGCCATT
Cyclin D3	NM_001136017	F: TACCCGCCATCCATGATCG
		R: AGGCAGTCCACTTCAGTGC
GAPDH	NM_002046	F: GAAGGTGAAGGTCGGAGTC
		R: GAAGATGGTGATGGGATTTC

F: forward; R: reverse.

Table 2. Synthetic system of PCR

Components	Volume per reaction
SYBR Green Super mix	5 μl
Forward primer (10 μM)	0.3-0.55 μl (270-450 nM)[2]
Reverse primer (10 μM)	0.3-0.55 μl (270-450 nM)
cDNA template	75-100 ng
Nuclease-free water	Up to 10 μl

In this study, we analyzed the effects of curcumin on miRNAs expression profile in human osteosarcoma cell line MG-63 and explored its mechanism.

Materials and methods

Cell culture and transfection

Human osteosarcoma cell line MG-63 was cultured with Eagle's Minimum Essential Medium (ATCC-30-2003) containing 10% fetal calf serum at 37°C with 5% CO_2. Transfection of miRNA mimic/inhibitor was performed using Amaxa Nucleofactor according to the manual. The foreign miRNA mimic/inhibitor was imported into the nucleus directly.

Detection of miRNA chip

The treated cells were harvested and counted, they were digested and miRNAs were isolated using miRNA isolation kit (mirVana, AM1561) according to the manual. The concentration and purity of miRNAs were detected with Qubit fluorometer. RNA was labeled Hy3 using Cancer MicroRNA Array kit (Signosis, AP-0003) according to the manual and hybridized in miRCUPY-TM LNA chips. Microarray images were scanned using GeneChipR Scanner 3000 and analyzed using miRNA QC Tool software.

RNA extraction and real-time PCR

The harvested cells were washed with RNase free PBS. Total RNA and miRNAs were extracted using miRNA isolation kit and RNeasy Mini Kit (Qiagen) respectively according to the manufacturer's protocol. Their concentration and purity were detected with Qubit fluorometer. 1 μg RNA was subjected to reverse transcription using reverse transcription kit (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qiagen). At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimmers. The primers used in this study were shown in **Table 1**, the synthetic system of PCR were shown in **Table 2**. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the $2^{-\Delta\Delta Ct}$ method.

MTT assay

The cells were treated with 0 μM, 10 μM, 20 μM and 40 μM curcumin respectively, the tested cells were seeded at density 5000 cells/well in 24-well plates and measured in 1, 2 and 3 days after culture. Before measured the cells were added 20 μl MTT (5 mg/ml) and the cells were incubated for an additional 4 h at 37°C. The culture medium was removed, 100 ml of DMSO were added to each well. With shaking at low speed for 10 min, the MTT solution was aspirated and optical densities (OD) of the supernatant were read at 492 nm using a Microplate Reader (Thermolex, Molecular Device Co). The experiments were repeated three times and the negative control was conducted using only cell-free culture medium (means ± SD).

Cell invasion

Matrigel was melt at 4°C overnight and diluted with pre-cooling Eagle's Minimum Essential medium to final concentration of 1 mg/ml. The 100 μl of 1 mg/ml Matrigel was added into the center of the upper chamber bottom of Transwell and incubate at 37°C to gelatine. 200 μl Eagle's Minimum Essential medium was added to each well for gel reconstruction. The cells were detached, suspended, counted and

Table 3. The 440 nm OD of MG-63 treated with different concentration of curcumin ($\overline{x}$±SD)

Time (h)[2] \ Curcumin (μM)	0	10	20	40
0	0.66±0.04			
24	0.74±0.02	0.51±0.03	0.41±0.03*	0.34±0.01*
36	0.62±0.04	0.36±0.09*	0.32±0.03*	0.24±0.01*
48	0.53±0.06	0.31±0.06*	0.21±0.06*	0.10±0.01**

*P<0.05, **P<0.01 *VS* control group.

Table 4. Curcumin treatment induced differential expressed miRNAs (24 h)

ProbeSet_Name	Fold_change (B1_vs_A1)	Regulation (B1_vs_A1)	median CV (%)	label
hsa-miR-149	2.4357	Up	5.32	Cy3
hsa-miR-138	2.6614	Up	6.12	Cy3
hsa-miR-181b	1.8996	Up	6.34	Cy3
hsa-miR-193b	2.0147	Up	6.58	Cy3
hsa-miR-339-5p	2.1366	Up	7.11	Cy3
hsa-miR-22	1.9654	Up	6.04	Cy3
hsa-miR-671-5p	1.7452	Up	6.23	Cy3
hsa-miR-124	1.8639	Up	5.99	Cy3
hsa-miR-744	2.1660	Up	6.10	Cy3
hsa-miR-455-3p	1.8254	Up	6.38	Cy3
hsa-miR-494	1.6332	Down	7.46	Cy3
hsa-miR-186	2.4365	Down	8.20	Cy3
hsa-miR-100	2.3334	Down	6.09	Cy3
hsa-miR-154	1.7562	Down	7.13	Cy3

A1: DMSO control group, B1: 20 μM Curcumin group.

cultured in upper chamber of Transwell, the lower chambers of Transwell were filled with medium with 10% FBS and incubated at 37°C. The liquid in upper chambers was discarded and the upper chambers were taken out. The cells not passing through the membrane were wiped off with cotton swab. The chambers were fixed with 4% paraformaldehyde at room temperature for 10 min and stained with 0.1% crystal violet. The cells were observed and counted under high magnification microscopy.

Protein extraction and Western blotting determination

The cells were lysed with RIPA lysis buffer and total proteins of cells were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaked at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to GAPDH.

Statistical analysis

The results are expressed as mean ± S.D. and analyzed with SPSS 11.5 software; t-test was used to evaluate the differences between groups. A value of *P*<0.05 and *P*<0.01 was taken to denote statistical significance.

Results

Effects of curcumin on the growth of MG-63 cells

The proliferation of MG-63 cells treated by 0 μM, 10 μM, 20 μM and 40 μM curcumin was detected by MTT method after treatment for 24 h, 36 h and 48 h respectively. The results were shown in **Table 3**. We found that curcumin could significantly inhibit the proliferation of MG-63 cells (P<0.05).

Effects of curcumin on the miRNAs expression profile in MG-63 cells

The effects of curcumin on the miRNAs expression profile in MG-63 cells were detected by miRNA chips. More than 1.5 times of differen-

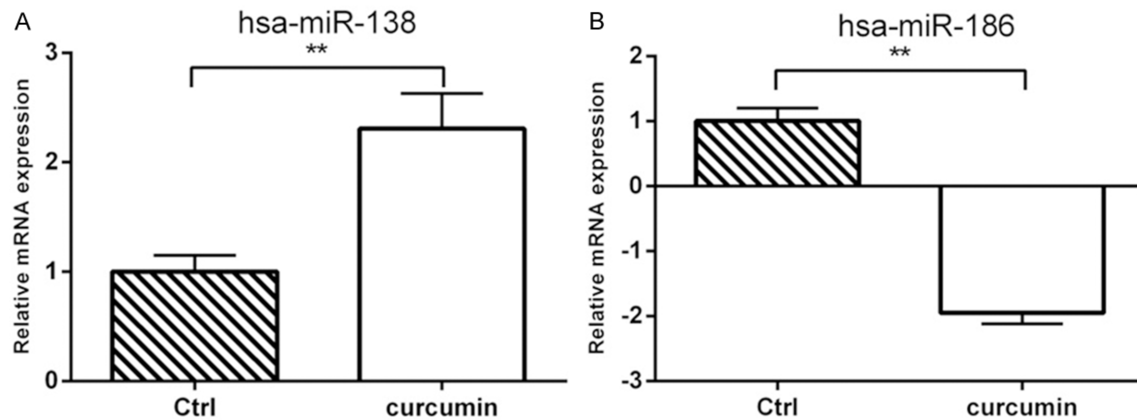

Figure 1. Effects of curcumin on the expression of hsa-miR-138 and hsa-miR-186 in MG-63 cells. A: hsa-miR-138; B: hsa-miR-186. **P<0.01.

Table 5. Prediction of miRNA targets

MicroRNA	Gene	RefseqID	Seed Length	Start	Sequence	End	Region	*P* value
hsa-miR-138	LIPH	NM_139248	11	2076	AGCUGGUGUUG	2066	3 UTR	0.0002
	PDE3A	NM_000921	11	3663	AGCUGGUGUUG	3653	3 UTR	0.0002
	KIAA0494	NM_014774	11	5476	AGCUGGUGUUG	5466	3 UTR	0.0008
	CCND3	NM_001136017	11	3305	AGCUGGUGUUG	3295	3 UTR	0.0006
	TSR1	NM_018128	11	3863	AGCUGGUGUUG	3853	3 UTR	0.0004
	CEBPG	NM_001806	11	2542	AGCUGGUGUUG	2532	3 UTR	0.0007
	SMAD4	NM_005359	11	1611	AGCUGGUGUUG	1601	3 UTR	0.0004
	MEOX1	NM_004527	11	2054	AGCUGGUGUUG	2044	3 UTR	0.0004
	YBX2	NM_015982	10	1278	AGCUGGUGUU	1269	3 UTR	0.0004
	DGKG	NM_001346	10	2934	AGCUGGUGUU	2925	3 UTR	0.0028
	RELA	NM_001145138	10	1443	AGCUGGUGUU	1434	3 UTR	0.0014
	ZAK	NM_133646	10	6184	AGCUGGUGUU	6175	3 UTR	0.0054

tially expressed miRNAs after the treatment of 20 μM curcumin for 24 h were shown in **Table 4**. The larger changed miRNAs were selected to confirm the chip results. Real-time PCR results of hsa-miR-138 and hsa-miR-186 were shown in **Figure 1**. It showed that hsa-miR-138 significantly increased after the treatment of 20 μM curcumin for 24 h while hsa-miR-186 significantly decreased after the treatment of 20 μM curcumin for 24 h.

Bioinformatics analysis of miRNA target genes

miRNA target gene prediction software of PicTar and TargetScan were used to predict the miRNA target genes. Some predicted target genes in the intersection of these two softwares were shown in **Table 5**. Cell differentiation and proliferation related genes Smad4, NFκB p65 (RELA) and cyclin D3 were selected from these genes.

Effects of curcumin on the expression of miRNA138 and its target genes

Western blotting results showed that curcumin significantly inhibit the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells. RT-PCR results showed that compared with curcumin group, overexpression of hsa-miR-138 could down-regulate the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells, while hsa-miR-138 inhibitor could up-regulate the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells (**Figure 2**).

Effects of curcumin on the invasion ability of MG-63 cells

The results of cell invasion experiment were shown in **Table 6**. It showed that cell numbers

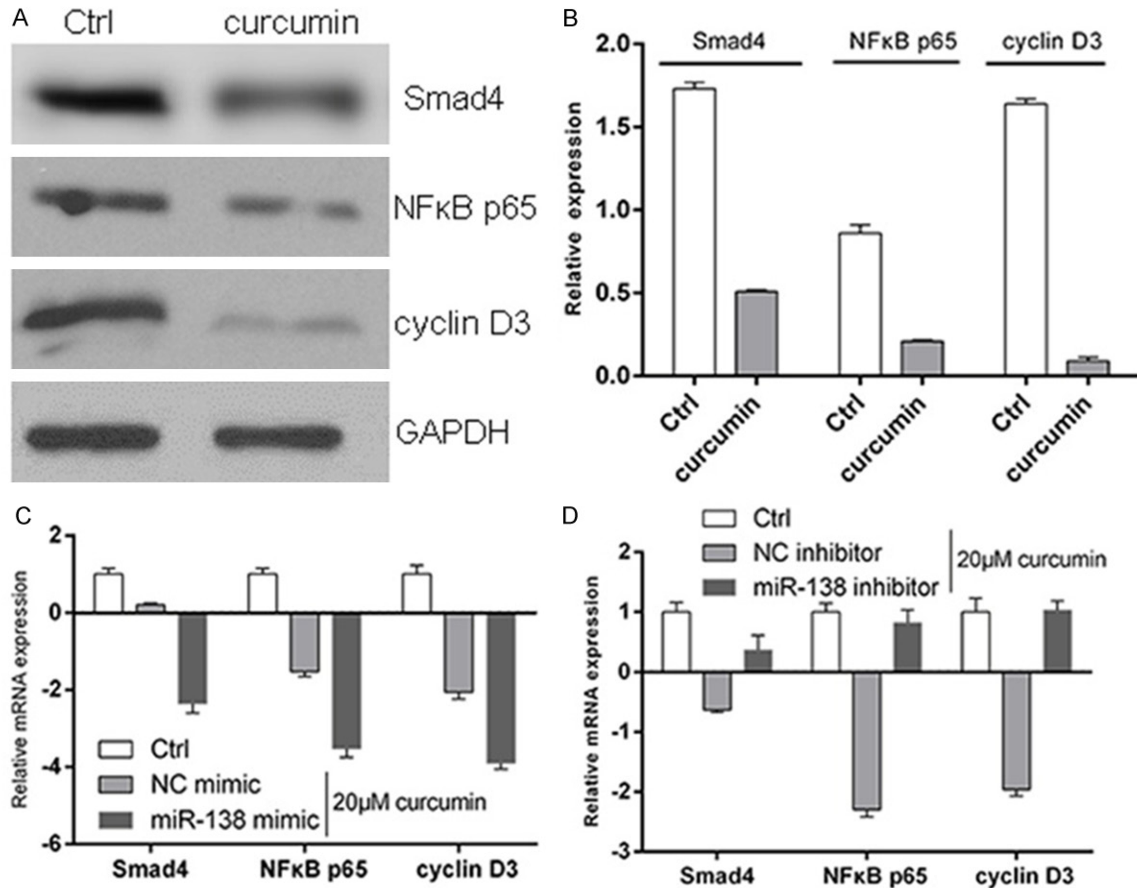

Figure 2. Effects of curcumin on the expression of miRNA138 and its target genes. A, B: Western blotting results showed that curcumin significantly inhibit the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells; C: miR-138 mimic could down-regulate the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells; D: miR-138 inhibitor could up-regulate the expression levels of Smad4, NFκB p65 and cyclin D3 in MG-63 cells.

across the membrane decreased after the treatment of curcumin. The invasion ability of MG-63 cells decreased after the transfection of miR-138 mimic, while the invasion ability enhanced after the transfection of miR-138 inhibitor ($P<0.05$).

Discussion

miRNAs has a variety of biological functions and its mechanism is also very complex. miRNAs and its target genes or other miRNAs combine to form a complex regulatory network to involve in the life processes, especially in the development of tumors [12-14]. A variety of abnormal expression of miRNAs was found in the osteosarcoma [8-11]. The application of miRNA chip makes it possible for large-scale screening of miRNA profile. The target genes of miRNA can be predicted by bioinformatics method after the abnormal expression of miRNAs was detected [15]. There are six miRNA target gene prediction softwares (miRanda; TargetScan; RNAhybrid; PicTar; RNA22; MicroInspector) used widely. The intersection of PicTar and TargetScan predicted well for the miRNA function site in 3'UTR region [16].

In this study, we screened the abnormal expression of miRNAs in MG-63 cells after the treatment of curcumin and explored the effects of curcumin on MG-63 cells and its mechanism using Affemitrix miRNA chip and bioinformatics analysis. We found that hsa-miR-138, hsa-miR-149, hsa-miR-181b, hsa-miR-193b, hsa-miR-339-5p, hsa-miR-671-5p, hsa-miR-22 and hsa-miR-124 up-regulated and hsa-miR-494, hsa-miR-186, hsa-miR-100 and hsa-miR-154 down- regulated after the treatment of curcumin. Smad4, NFκB p65 and cyclin D3 were predicted as the target genes of hsa-miR-138 by bioinformatics analysis. Smad4 is an intermedi-

Table 6. Results of cell invasion Experiment

	Blank Control	miRNAs mimic		miRNAs inhibitor	
		20 μM curcumin			
		Control	miR-138	Control	miR-138
V1[a]	172[b]	122	96	134	186
V2	178	137	99	120	193
V3	194	129	108	117	182
	181.33±11.37[c]	129.33±7.51	101.00±6.24**	123.67±9.07	187.00±5.57**

[a]: Field of vision; [b]: cell numbers through the basement membrane; [c]: Mean ± SD. Compared with control, **$P<0.01$.

ate in the TGF-beta signal pathway and abnormally expressed in many tumors, it could enhance the migration ability of tumor cells [17, 18]. P65 is a member of the NF-κB transcription factor protein family. When cells are stimulated by extracellular signals, the activation IκB kinase (IKK) makes the phosphorylation of IκB and the nuclear localization site of NF-κB exposed. Free NF-κB shifted to the nucleus and combined with specific κB sequences to induce the transcription of related genes [19]. Cyclin D3 is an important regulatory factor from G1 to S phase of cell cycle, studies showed that it was the adverse prognostic factor of diffuse large B-cell lymphoma (DLBCL) [20]. We detected the expression levels of Smad4, NFκB p65 and cyclin D3 with western blotting method and found that they down-regulated after the treatment of curcumin. Transient transfection of hsa-miR-138 mimic and hsa-miR-138 inhibitor to change the expression of hsa-miR-138 showed that overexpression of hsa-miR-138 down-regulated the expression levels of Smad4, NFκB p65 and cyclin D3 compared with the treatment of curcumin, while inhibition of hsa-miR-138 up-regulated the expression levels of Smad4, NFκB p65 and cyclin D3. These suggested that curcumin could increase the expression of hsa-miR-138, hsa-miR-138 inhibited cell proliferation and invasive ability by inhibition of its target genes. This conclusion was also confirmed by Transwell cell invasion assay.

In a word, curcumin could increase the expression of hsa-miR-138, hsa-miR-138 inhibited cell proliferation and invasive ability by inhibition of its target genes.

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Disclosure of conflict of interest

None.

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