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Original Article

Inhibition of curcumin on proliferation and invasion in giant cell tumor of bone (GCTB) by targeting STAT3

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Abstract: Objectives: To investigate the effects of curcumin on the proliferation and invasion in cells of giant cell tumor of bone (GCTB) by targeting the signal transducer and activator transcription factor-3 (STAT3), in order to supply treatments and preventions on GCTB with new drugs and relative mechanisms. Methods: GCTB cells were collected from resection tissues of four patients with GCTB. GCTB cells were treated by curcumin. STAT3 was one of the genes influencing invasion of osteolytic tumor, so we silenced STAT3 by small interfering RNA (siRNA) and detected by Western Blot (WB). Cell counting kit-8 (CCK-8) and Transwell system were used for detecting GCTB cell proliferation and invasion. Results: Curcumin (31.5 nM) was treated on GCTB cells for 24 h. There was significant difference before (case 1, 0.685±0.026; case 2, 0.731±0.030; case 3, 0.728±0.019; case 4, 0.634±0.015) and after (case 1, 0.160±0.008; case 2, 0.769±0.014; case 3, 0.743±0.026; case 4, 0.750±0.018) curcumin treatment ($P<0.01$). After silencing STAT3, there was no significance before (case 1, 0.738±0.021; case 2, 0.769±0.014; case 3, 0.743±0.026; case 4, 0.750±0.018) and after (case 1, 0.704±0.010; case 2, 0.720±0.015; case 3, 0.714±0.022; case 4, 0.729±0.013) curcumin treatment. Conclusions: Curcumin inhibits the proliferation and invasion in GCTB by targeting STAT3. STAT3 might be the new therapeutic target for treating GCTB metastasis.

Keywords: Giant cell tumor of bone, STAT3, curcumin, proliferation, invasion

Introduction

Giant cell tumor of bone (GCTB) is the rare primary osteolytic tumor of bone, which caused massive bone destruction at the epiphysis of long bones [1]. Histologically, GCTB was complex involving neoplastic ovoid mononuclear cells sheets with high level of RNAK ligand (RANKL), myeloid cells with positive RNAK, and the osteoclast-like giant cells with large RNAK expression [2-5]. According to Goldring et al. [6] and Thomas et al. [5], the giant cell of GCTB was osteoclastic in nature, while the true neoplastic cells were those appearing ovoid and displaying markers of mesenchymal stem cells with partial differentiation along the osteoblast lineage [7-9]. This tumor, with the hallmarks of the aggressively lytic behavior and as the role of the osteoclast-like tumor giant cells in the lytic process, contained small areas of osteoid matrix deposition, woven bone and occasionally new bone [10, 11], as the reactive tissue at tumor margin or the forming within the tumor. Traditionally, the surgery for treating GCTB was usual but often associated with high recurrence and morbidity. Sometimes there needed to amputate. However, there was less reported about the drug therapy for GCTB. According to previous reports [12, 13] showed that denosumab was the with efficiency for treating GCTB by influencing RANK/RANKL pathway in the pathogenesis of this disease.

Recently, many traditional Chinese for treating on and preventing from different kinds of tumor draws more and more attentions. Curcumin (diferuloylmethane), as the yellow pigment of turmeric abundant in Southeast Indian food, was the pharmacologically safe polyphenol derived from Curcuma longa (Linn), which was with anti-tumor effects in several types of cancers [14, 15]. Oral intake of curcumin showed

the beneficial effect against precancerous lesions in Phase I and II clinical trials [16, 17]. Plentifully previous investigations found that the curcumin could affect several target proteins and signaling pathways, such as AMPK-COX-2 [18], JNKs [19], E2F4 [20], cyclin D1-CDK-4 [21] and so on. Although numerous proteins have been reported as the targets of curcumin, the signal transducer and activator transcription factor-3 (STAT3), as the main protein in Jak-STAT3 signal pathway, has not been suggested as the direct target of curcumin, even in GCTB.

STAT signal pathway could be activated by more than 35 cytokines and growth factors, which was the critical role in widely cellular functions in hematopoietic, immunologic, neuronal, hepatic system and so on [22-24]. There were seven proteins, including STATs 1, 2, 3, 4, 5a, 5b and 6, mediating the signal transduction from extracellular signals to the target genes transcription [25, 26]. Numerous researches [26, 27] showed that STAT3 pathway was constitutively activated in many human tumors, including breast cancer, prostate cancer, cervical cancer , prostate cancer, colon cancer, melanoma, leukemia, multiple myeloma and so on. However, there was no investigation on the STAT3 expression in GCTB.

In our study, we used curcumin as the anti-tumor drug to treat on GCTB cells and observe the STAT3 expression and the effect of curcumin on the proliferation and invasion of GCTB, in order to provide clinical therapy on GCTB with new anti-tumor drug and target.

Methods and materials

GCTB tumor tissues collection

Human GCTB tissue and normal cancellous bone without bone marrow cells were collected from 4 patients with GCTB after resection in Department of Orthopaedics in Nan Fang Hospital from January, 2014 to December, 2014. The 4 patients were with primary tumor surgery and received resection by the same surgeon. The pathological analysis on GCTB tumor tissues was completed and reached consistency by two pathologists with experiences over than 10 years.

GCTB cell separation and culture

According to previous description [28], the fresh GCTB tumor tissues from the 4 cases were separated and cultured with dulbecco's minimum essential medium (DMEM, Gibco, Thermo Fisher Scientifics Inc. Shanghai, China) containing 10% fetal bovin serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin, (Gibco). The GCTB cells were cultured at 37°C, 5% CO_2 and saturated humidity. After the confluence reaching 90%, digested the primary GCTB cells with trypsin (Gibco) and passaged for amplification. Saved parts of the primary GCTB cells in liquid nitrogen, and the others were subculture. The GCTB cells in logarithmic phase were used for the following experiments.

Cytotoxicity and influence of curcumin on GCTB cell and STAT3 expression

Curcumin (Sigma-Aldrich Co. Shanghai, China) was dissolved in Dimethyl Sulphoxide (DMSO, Sigma) with the primary concentration of 500 mM and stored at 4°C. Curcumin was diluted in cell culture medium, as the working concentration in treating on GCTB cells. GCTB cells were treated with 10~200 nM for 24 hours. 0.1% DMSO was taken as negative control (NC). CCK-8 (Thermo) was used to detect the proliferation of GCTB cells after curcumin treatments and WB was for STAT3 expression.

Silencing and detecting STAT3 by siRNA and WB

siRNA directed against STAT3 was synthesized by Genepharma Company (Suzhou, China). The siRNA sequences targeting STAT3 were followed: sense 5'-CAU CUG CCU AGA UCG GCU AdTdT-3', anti-sense 5'-UAG CCG AUC UAG GCA GAU GdTdT-3'. siRNA was dissolved in Opti-MEM (Thermo) and mixed with Lipo-3000 (Thermo) according to the specifications. The mixture was treated on GCTB cells for 48~72 h.

The proteins with or without siRNA treatment were extracted with RIPA buffer (Thermo) and analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with 10% separating gel and 5% stocking gel. After transferring to PVDF (polyvinylidene fluoride, Merck Millipore Corporation, Shanghai, China) membrane, the PVDF membrane was blocked with 5% non-fat milk and 2% BSA (bovine serum albumin, Solarbio Life Sciences, Beijing, China) at 25°C for 2 h. Then the membranes were incubated with mouse anti-STAT3 monoclonal antibody (1:4000, Protientech Group Inc. Wu-

Table 1. Curcumin cytoxicity (0~100 nM) on GCTB cells in patient 1 (X±S)

Groups	OD value
NC	0.377±0.023
20 (nM)	0.259±0.014#
30 (nM)	0.132±0.021*
30.5 (nM)	0.130±0.020*
31 (nM)	0.130±0.005*
31.5 (nM)	0.125±0.015*
32 (nM)	0.125±0.008*
32.5 (nM)	0.124±0.014*
33 (nM)	0.122±0.004*
33.5 (nM)	0.123±0.016*
34 (nM)	0.122±0.019*
34.5 (nM)	0.120±0.007*
35 (nM)	0.121±0.020*
50 (nM)	0.119±0.018*
100 (nM)	0.116±0.005*

Note: GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; NC, negative control (0.1% DMSO); OD, optical density; #comparing to the NC, P<0.05; *comparing to the NC, P<0.01.

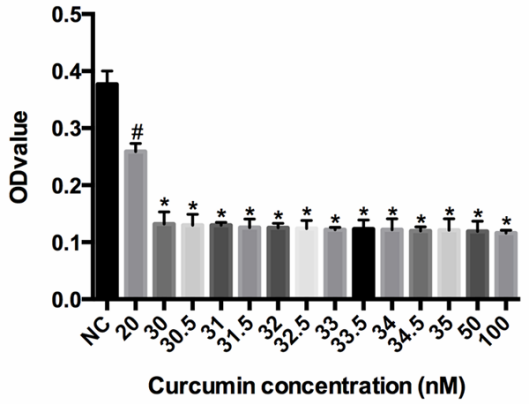

Figure 1. Curcumin cytotoxicity (0~100 nM) on GCTB cells. GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; NC, negative control (0.1% DMSO); #comparing to the NC, P<0.05; *comparing to the NC, P<0.01.

han, China) and rabbit anti-β-actin polyclonal antibody (1:10000, Proteintech), as internal parameter, at 4°C overnight. Goat anti-Rabbit and goat anti-mouse secondary antibodies (1:3000, Jackson ImmunoResearch Laboratories, Inc. USA) were used to incubate the membrane at 25°C for 1 h. ECL (electrochemiluminescence, Millipore) system was used for exposure.

GCTB cell proliferation assay

GCTB cells were cultured (4×10^3 cells/well) in 96-well plates for 24 h. Then the GCTB cells were treated with curcumin for 24 h. Cell proliferation was detected with CCK-8 by incubating for 40 min at 37°C in 5% CO_2 and saturated humidity. The absorbance was read at 450 nm.

GCTB cell invasion assay

40 μl of Matrigel (Becton, Dickinson and Company, BD, USA) was used to spread in the upper chamber of the Transwell system (BD). GCTB cells (1×10^5 cells/mL) with curcumin treatment for 24 h were cultured in DMEM without FBS and transferred to the upper chamber, while the lower chamber was with DMEM containing 10% FBS. After culturing for 18 h, cleaned the Matrigel and the GCTB cells in the upper chamber totally. Fixed the GCTB cells with 4% paraformaldehyde (Solarbio) for 30 min. Stained the GCTB cells with 0.25% crystal violet (Macklin Inc. Shanghai, China) in 20% methanol (Solarbio) for 20 min. Observed the GCTB cells transferred to the lower surface of the upper chamber. Randomly selected 5 fields (20×) and calculated the number of the GCTB cells. The average was obtained and compared.

Statistical analysis

All the data were analyzed by SPSS 21.0 software and showed as mean ± standard deviations (X±S). One-Way ANOVA was for comparisons between different treatments. Least Significant Difference (LSD) was for variance homogeneity, and Dunnett's T3 was for variance heterogeneity. A value of $P<0.01$ considered significant difference.

Results

Final concentration of curcumin treating on GCTB cells

Curcumin treatment (10~200 nM) on GCTB cells was detected by orthogonal design and cytotoxicity assay. As showed in **Table 1** and **Figure 1**, the different concentration of curcumin treating on GCTB cells had different effects on GCTB cell proliferation. When curcumin concentration above 31.5 nM, the proliferation of GCTB cells was with significance comparing to

Table 2. Relative gray value of STAT3/β-actin in GCTB cells with curcumin cytotoxicity (0~100 nM) assay

Groups	Relative gray value
NC	0.893±0.036
20 (nM)	0.704±0.027#
30 (nM)	0.538±0.027*
30.5 (nM)	0.421±0.029*
31 (nM)	0.414±0.030*
31.5 (nM)	0.408±0.020*
32 (nM)	0.406±0.014*
32.5 (nM)	0.406±0.025*
33 (nM)	0.402±0.016*
33.5 (nM)	0.404±0.018*
34 (nM)	0.401±0.015*
34.5 (nM)	0.402±0.012*
35 (nM)	0.400±0.017*
50 (nM)	0.387±0.020*
100 (nM)	0.380±0.018*

Note: GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; NC, negative control (0.1% DMSO); #comparing to the NC, P<0.05; *comparing to the NC, P<0.01.

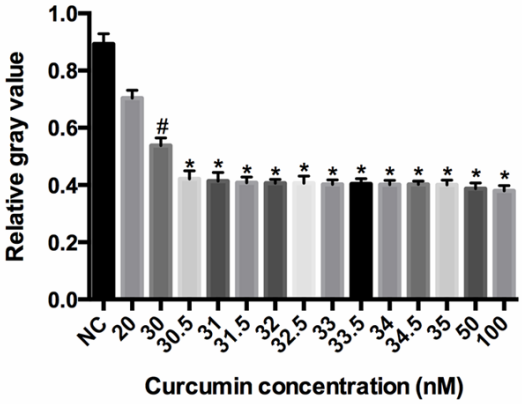

Figure 2. Relative gray value of STAT3/β-actin in GCTB cells with curcumin cytotoxicity (0~100 nM) assay. GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; NC, negative control (0.1% DMSO); #comparing to the NC, P<0.05; *comparing to the NC, P<0.01.

the NC (P<0.01) and tended to be stable. Therefore, we chose 31.5 nM of curcumin as the final concentration for curcumin treatment on GCTB cells. As showed in **Table 2** and **Figure 2**, with 31.5 μM of curcumin treatment, STAT3 expression was inhibited comparing to the NC, significantly (P<0.05).

Proliferation of GCTB cells with/without curcumin treatment for 24 h and/or silencing STAT3

31.5 nM of curcumin was used to treat on GCTB cells with silencing STAT3 for 24 h. GCTB cells proliferation were detected and compared. As showed in **Table 3** and **Figure 3**, there was significant difference of transferred GCTB cells before and after curcumin treatment (31.5 nM) (P<0.01), while no significance before and after curcumin treatment combined with silencing STAT3. These results showed that when treating with curcumin (31.5 nM), in the four cases, both of the GCTB cells proliferations were both inhibited. After silencing STAT3, the curcumin (31.5 nM) treatment could not inhibit the GCTB cells proliferations, which indicated that curcumin could inhibit GCTB cell proliferation by targeting STAT3.

Invasion of GCTB cells with/without curcumin treatment for 24 h and/or silencing STAT3

31.5 nM of curcumin was used to treat on GCTB cells with silencing STAT3 for 24 h. GCTB cells invasion were detected and compared. As showed in **Table 4** and **Figure 4** there was significant difference of transferred GCTB cells before and after curcumin treatment (31.5 nM) (P<0.01) (**Figure 4Ba** and **4Bb**), while no significance before and after curcumin treatment combined with silencing STAT3 (**Figure 4Ba** and **4Bc**). These results showed that when treating with curcumin (31.5 nM), in the four cases, both of the GCTB cells invasions were both inhibited. After silencing STAT3, the curcumin (31.5 nM) treatment could not inhibit the GCTB cells invasions, which indicated that curcumin could inhibit GCTB cell invasion by targeting STAT3.

Discussions

GCTB was generally a benign but locally aggressive tumor with metastatic and invasive ability, which was found to spread to the lungs [29, 30]. At present, there was no reliable predictor for the recurrence or metastasis of GCTB. The most common therapy was resection. However, one-forth of the patients would suffer the recurrence and one-half of them would need a joint-sacrificing segmental resection [31]. In our study, we used curcumin as the therapeutic drug to treat on GCTB cells, which were obtained

Table 3. Proliferation of giant cell tumor of bone cells with/without curcumin treatment (31.5 nM) for 24 h and/or silencing STAT3 (OD value) (X±S)

	Curcumin treatment		Curcumin treatment combined with silencing STAT3	
	Before	After	Before	After
Case 1	0.685±0.026	0.160±0.008*	0.738±0.021	0.704±0.010
Case 2	0.731±0.030	0.178±0.011*	0.769±0.014	0.720±0.015
Case 3	0.728±0.019	0.157±0.012*	0.743±0.026	0.714±0.022
Case 4	0.634±0.015	0.165±0.015*	0.750±0.018	0.729±0.013

Note: GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; *comparing to the "before", P<0.01. (Would you mean that this table should be deleted?).

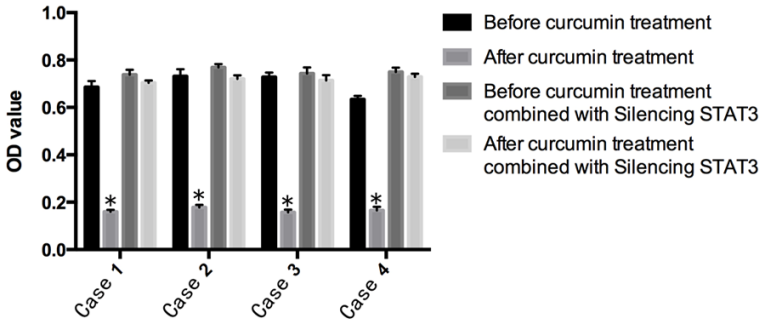

Figure 3. Proliferation of giant cell tumor of bone cells with/without curcumin treatment (31.5 nM) for 24 h and/or silencing STAT3 (OD value); GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; Case 1, patient 1; Case 2, patient 2; Case 3, patient 3; Case 4 patient 4; *comparing to "Before curcumin treatment", P<0.01. (The curcumin treatment could inhibit the proliferation of GCTB cells in the four cases. When silencing STAT3 and then treating with curcumin, the proliferation of GCTB was not inhibited, which was similar to before silencing and treatments. This indicated the STAT3 was the target gene in GCTB cell with curcumin treatment. Compared the group "Before curcumin treatment" with "After curcumin treatment", as well as group "Before curcumin treatment combined with silencing STAT3" with "After curcumin treatment combined with silencing STAT3", were more suitablely and directly on showing the effect of curcumin the target gene STAT3. If these were not suitable, please tell us how to show the data and how to compare the result.).

Table 4. Invasion of giant cell tumor of bone cells with/without curcumin treatment (31.5 nM) for 24 h and/or silencing STAT3 (cell counting) (X±S)

	Curcumin treatment		Curcumin treatment combined with Silencing STAT3	
	Before	After	Before	After
Case 1	130.20±12.88	46.53±9.70*	125.64±10.09	117.94±9.01
Case 2	132.96±13.06	44.60±10.93*	124.83±9.56	122.56±10.68
Case 3	129.40±13.25	41.38±12.09*	125.60±11.03	120.48±9.11
Case 4	130.09±10.52	41.55±8.76*	125.42±8.84	121.07±10.56

Note: GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; *comparing to the "before", P<0.01.

from 4 patients with tumor resection. By detecting and silencing STAT3 expression, we used CCK-8 and transwell assays to analyze the proliferation and invasion of the GCTB cells, in order to find the target of the traditional Chinese drug and provide clinical therapy with more molecular target and GCTB metastatic mechanisms.

In our study, GCTB cell proliferation was inhibited by treating with curcumin (31.5 nM), significantly (P<0.01). When silencing STAT3, curcumin had no obvious effect on GCTB cell proliferation. According to Roudier et al. [32], denosumab was the common drug for GCTB, and it could decrease proliferation in GCTB. However, the injection of denosumab was not suitable for the patients with multiple myeloma (MM) to prevent from skeletal related events, as well as easily induce side-effects, such as hypocalcemia [33], osteonecrosis [34], fatigue, weakness, hypophosphataemia [35], nausea and so on.

Curcumin, as the save and effective anti-tumor drugs, was with less side-effect. Curcumin intake is convenient, and the daily eatable curcumin is abundant in many foods. Curcumin was proven to inhibit metastasis in colorectal cancer [36], breast cancer [37] and so on. There was less evidence of curcumin on inhibiting GCTB metastasis. In our study, after treating with curcumin (31.5 nM), GCTB cell invasion was inhibited, while silencing STAT3 could reduce the inhibition of

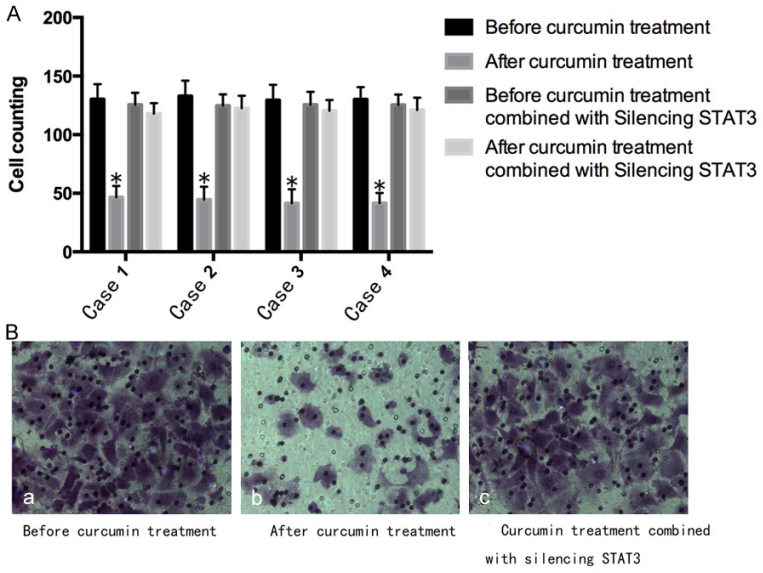

Figure 4. Invasion of giant cell tumor of bone cells with/without curcumin treatment (31.5 nM) for 24 h and/or silencing STAT3 (cell counting); GCTB, giant cell tumor of bone; STAT3, signal transducer and activator transcription factor-3; Case 1, patient 1; Case 2, patient 2; Case 3, patient 3; Case 4 patient 4; *comparing to "Before curcumin treatment", P<0.01.

curcumin on GCTB cells. These results indicated that curcumin could inhibit GCTB cell invasion, and STAT3 was the target protein of curcumin.

However, there were still some insufficiencies in our study. Firstly, there were only 4 samples of GCTB tumor obtained in our investigation. Secondly, the proliferation and invasion experiments were carried on in vitro, which would lead to the effect of curcumin being influenced by external factors. Last but not the least, though STAT3 expression was silenced, the concrete mechanism of curcumin influencing on GCTB cell proliferation and invasion was still unclear. Therefore, we need further investigations on the role of STAT3 in the inhibition of curcumin on GCTB cell proliferation and invasion.

Conclusively, curcumin could reduce the proliferation and invasion in GCTB by targeting STAT3. STAT3 might be the new target for treating GCTB invasion.

Disclosure of conflict of interest

None.

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