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ORIGINAL RESEARCH

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Curcumin enhances breast cancer cell death by suppressing PD-L1 expression

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Abstract

Background: To investigate the effects of curcumin on the growth of breast cancer (BC) cells *in vitro* and to explore the role of Programmed death ligand 1 (PD-L1) in this process. **Methods**: Curcumin's effects on the growth and apoptosis of MCF-7 and MDA-MB-231 breast cancer cells were assessed using cell counting kit-8 (CCK-8), flow cytometry and Western blotting. The inhibition of PD-L1 expression by curcumin was evaluated through co-transfection of PGL3-PD-L1 plasmids and luciferase activity. Additionally, the Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway was analyzed after treatment with curcumin and the PD-L1 inhibitor BMS202. **Results**: Curcumin had anti-proliferative and pro-apoptosis effects on human breast cancer cells. Curcumin significantly inhibited the expression level of both the PD-L1 protein and the PD-L1 mRNA in BC cells. The abundance of PD-L1 transcripts in the BC cells with curcumin decreased significantly, but increased with the increase in curcumin concentration. The activity of luciferase significantly decreased with the increase in concentration of the breast cancer cells transfected with PD-L1 promoter after treatment with curcumin. Curcumin also induced the inhibition of PD-L1 expression, and mediates the inhibition of the PI3K/AKT/mTOR axis. **Conclusions**: The combination of curcumin and PD-L1 inhibits the proliferation of BC cells and induces apoptosis, which may be related to curcumin's inhibition of the activity of PD-L1 promoter and the downstream PI3K/AKT/mTOR axis.

Keywords

Breast cancer; Curcumin; PD-L1; Luciferase report gene; PI3K/AKT/mTOR

1. Introduction

Curcumin is a phenolic pigment extracted from the rhizomes of herbaceous plant, turmeric. It has good antioxidant and anti-inflammatory effects [1], and a large number of cell tests and animal tests have proved that curcumin has a clear anti-tumor activity and has extensive tumor suppressor effect [2]. However, the mechanism of inhibiting the growth of tumor cells has not been fully studied.

Programmed death ligand 1 (PD-L1) is one of the ligand of programmed death factor 1 (PD1), which is expressed on the surface of a variety of tumor cells and immune cells [3, 4]. Through the combination of high expressions of PD-L1 and the receptor PD1 on the surface of tumor infiltrating lymphocyte, the tumor cells transferred the immunosuppressive signal to Tumor-Infiltrating Lymphocytes (TILs), inhibited the motility and proliferation of T cells, secreted the cytotoxic medium, induced the depletion of T cells and limited the killing effect of the cells to the tumor cells, thus realizing the escape of the immune system [3–5]. PD-L1/PD1 targeted immunotherapy is effective in the clinical trials of various tumors [4, 6], suggesting that the PD-L1/PD1 pathway is vital in tumor progression.

Studies have reported that targeted inhibition of PD-L1 expression can inhibit tumor cell proliferation and induce apoptosis, thereby inhibiting tumor progression. In this study, it was discovered that curcumin, as a widely added active plant extract with high safety, can effectively inhibit the growth as well as induce apoptosis of breast cancer cells, which provides a way as well as a variety of options to assist in the treatment of BC.

2. Materials and methods

2.1 Cell lines and major reagents

Human breast cancer cell lines MCF-7 and MDA-231 were purchased from the Shanghai Institute of Cell Research. Cell culture conditions: Roswell Park Memorial Institute (RPMI) 1640 (11875093, Gibco, Grand Island, NY, USA), 37 ℃ containing 10% fetal bovine serum and culture at 5% CO_2. CCK-8 assay kit (C0038, Beyotime, Beijing, China); Annexin V/Propidium Iodide (PI) apoptosis kit (C1090, Beyotime, Bei-

jing, China), β-Actin, PD-L1, PI3K, AKT and mTOR antibodies (ab8226, ab205921, ab189403, ab8805, ab32028, Abcam, Cambridge, UK).

2.2 CCK-8 cell survival assay

The rationale for the selected concentrations is based on previous studies as well as preliminary experiments conducted in our laboratory to ensure that the concentrations used are both physiologically relevant and effective [2]. Gradient concentration of curcumin (0, 1, 5, 10 μg/mL) were used for treatment. 10 μL of CCK-8 was added to each well, and the plate was incubated at 37 °C for an hour, and 450 nm Optical density (OD) value was measured.

2.3 Cell apoptosis detection

The cells were resuspended in annexin-V binding buffer, and then stained with 5 μL Annexin-V-Fluorescein Isothiocyanate (FITC) and 10 μL propidium-iodide (PI). Fluorescence was analyzed on a FACS spectrometer (antoTMII, BD Biosciences, San Jose, CA, USA).

2.4 Western blotting

Samples were dissolved by 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with primary antibodies. After immuno-blotting with secondary antibodies.

2.5 qPCR detection

To collect the breast cancer cells treated with curcumin with different concentrations, the total RNA was extracted by trizol, and the two-step method was used for reverse transcription and Polymerase Chain Reaction (PCR) amplification according to the operation of the quantitative Polymerase Chain Reaction (qPCR) Kit (TAKARA company, CN830A, Otsu, Japan). The primer sequences for *PD-L1* gene: forward: 5′-CAAAGAATTTTGGTTGTGGA-3′ and reverse: 5′-AGCTTCTCCTCTCTCTTGGA-3′. The primer sequences for *β-actin* gene: forward: 5′-TGGATCAGCAAGCAGGAGTATG-3′; reverse: 5′-GCATTTGCGGTGGACGAT-3′.

2.6 Determination of luciferase activity

The cells were seeded on 24-well plates according to the number of cells (1.5×10^5)/pore. After 24 h, when the cell fusion degree was greater than 90%, transfection was carried out with PGL3-PD-L1 1 μg, p-RL-TK 0.04 μg, Lipofectamine 2000 2 μL per pore, and 500 μL medium without antibiotic and serum free. 48 h after transfection, the cells were collected and luciferase activity was measured. The experimental procedure was carried out in accordance with the instructions of Dual-luciferase Assay System kit. The activity of firefly luciferase in PGL3 was determined in the luminescence apparatus, the reading number was M1; and the fluor luciferase activity in the internal reference plasmid was M2.

2.7 Statistical analysis

All values were expressed as the $\bar{x} \pm s$. Statistical analysis were carried out by one-way Analysis of Variance (ANOVA) performed using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA). p value < 0.05 was considered as statistically significant.

3. Results

3.1 Effect of curcumin on the proliferation and apoptosis of breast cancer cell line MCF-7 and MDA-231

To evaluate the *in vitro* effect of curcumin on BC cell line MCF-7 and MDA-231, the CCK-8 method was used (Fig. 1). Gradient concentration curcumin (0, 1, 5, 10 μg/mL) was used to deal with BC cell line for 24 h, 48 h and 72 h. Results showed that curcumin had anti-proliferative effects on BC cells, and the effects were revealed (Fig. 1A,B).

To test the *in vitro* effect of curcumin on the apoptosis of breast cancer cell line MCF-7, a flow cytometry experiment was conducted with gradient concentration curcumin (0, 1, 5, 10 μg/mL) which was treated for 24 h. The results showed that the apoptosis rate of the cells increased with the increase of the concentration of curcumin (Fig. 1C). The apoptosis rate of MCF-7 was 0.6%, 7.3%, 27.1%, 44.2% (Fig. 1C). Results also showed that curcumin had pro-apoptosis effects on human breast cancer cells.

3.2 Curcumin can significantly inhibit both the expression level of PD-L1 protein and PD-L1 mRNA in breast cancer cells

To investigate the inhibitory effect of curcumin on PD-L1, Western blotting was carried out. As shown in Fig. 2A, the expression of PD-L1 showed decreased expression with an increasing concentration of curcumin.

To assess the effect of curcumin on PD-L1 mRNA expression in breast cancer cells, cells were treated with different concentrations of curcumin for 24 hours. Total RNA was then extracted, and reverse transcription was performed to synthesize cDNA. PCR amplification of PD-L1 mRNA was carried out, and the amplified product was analyzed using 1% agarose gel electrophoresis. The results showed that Fig. 2B, the abundance of PD-L1 transcripts in the BC cells with curcumin decreased significantly, and the decrease was altered with the increase of curcumin concentration. Fig. 2C,D further indicated the quantification of Fig. 2A,B. Therefore, Curcumin can significantly inhibit both the expression level of PD-L1 protein and PD-L1 mRNA in breast cancer cells.

3.3 Effect of curcumin on the activity of PD-L1 promoter in breast cancer cells

In the recombinant plasmid PGL3-PD-L1, luciferase expression is regulated by the upstream PD-L1 promoter region. After the transfection of PGL3-PD-L1 into BC cells, luciferase activity directly reflected the intensity of the promoter action. PGL3-PD-L1 and the internal control plasmid were co-transfected to BC cells. After 6 h was added to 24 h of different concentrations of curcumin, the activity of luciferase

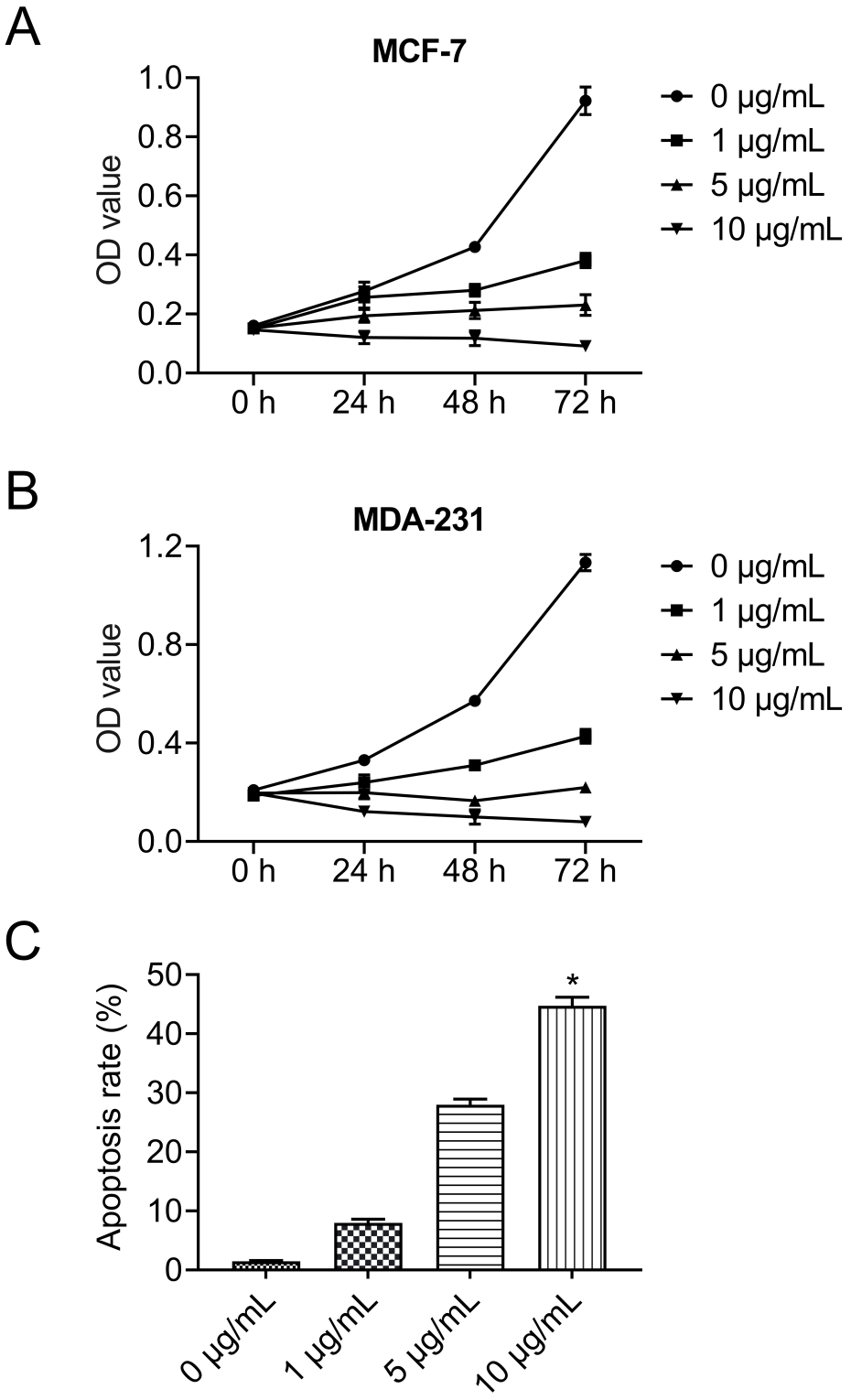

FIGURE 1. Effect of curcumin on the proliferation and apoptosis of breast cancer cell line MCF-7 and MDA-231. CCK-8 method was used to test the cell viability. Gradient concentration curcumin (0, 1, 5, 10 μg/mL) was used to deal with MCF-7 (A) and MDA-231; (B) Results showed that curcumin had anti-proliferative effects on human breast cancer cells, and the effects were in a concentration dependent manner. Flow cytometry experiment with gradient concentration curcumin (0, 1, 5, 10 μg/mL) treated was used to detect the apoptosis rate. Results showed that the apoptosis rate of the cells increased with the increase of the concentration of curcumin, and the effect was concentration dependent; (C) The apoptosis rate of MCF-7 was 0.6%, 7.3%, 27.1%, 44.2%. OD: Optical density.

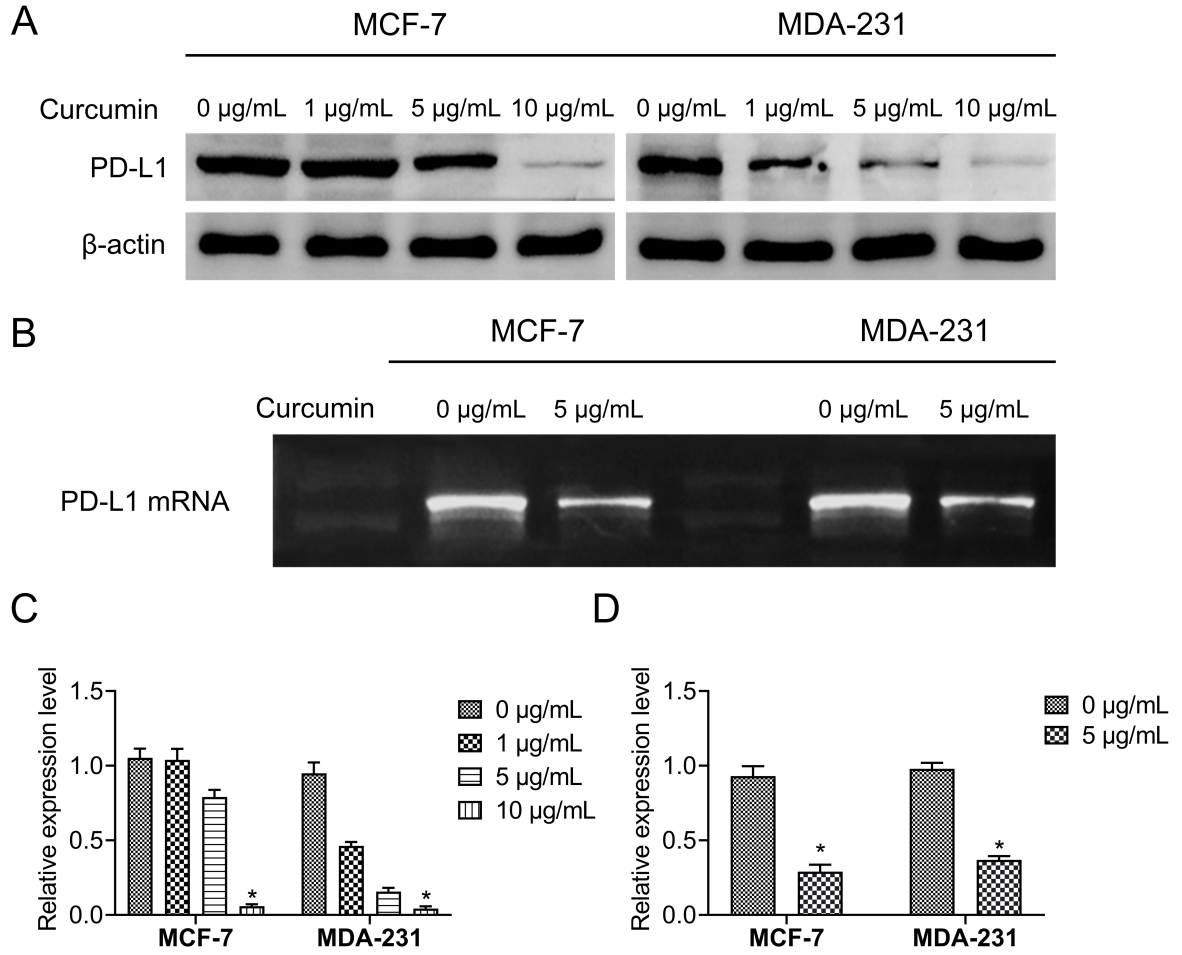

FIGURE 2. Curcumin can significantly inhibit both the expression level of PD-L1 protein and PD-L1 mRNA in breast cancer cells. Western blotting was carried out to investigate the inhibitory effect of curcumin on PD-L1. The expression of PD-L1 protein showed decreased expression along with the concentration of curcumin increasing; (A,C) RT-PCR was used to determine the effect of curcumin on the expression of PD-L1 mRNA. Compared with the untreated control, the abundance of PD-L1 transcripts in the breast cancer cells with curcumin decreased significantly, and the decrease was increased with the increase of curcumin concentration (B,D). *$p < 0.05$. PD-L1: Programmed Death-Ligand 1.

expression was determined by Dual-luciferase Assay System. The results showed that the activity of luciferase significantly decreased with the increase of concentration, and the prolongation of the phase of the breast cancer cells transfected with PD-L1 promoter after treatment with curcumin (Fig. 3A). 10 μg/mL curcumin had the most obvious effect, and after 24 h, the activity of BC cell activity decreased by 3.36 and 5.45 times respectively (Fig. 3A).

3.4 Curcumin combined with PD-L1 inhibitor can enhance the effect of killing breast cancer cells

In order to further study the correlation between the killing effect of curcumin on breast cancer cells and PD-L1, 5 μg/mL curcumin and 20 nM PD-L1 inhibitor BMS202 was used to act on the breast cancer cell MCF-7, and the flow cytometry was used to determine the apoptosis level of the breast cancer cells after the drug treatment. The experimental results showed that 5 μg/mL curcumin, (20 nM) PD-L1 inhibitor BMS202 and curcumin 5 μg/mL + BMS202 (20 nM) treated 24 h, the apoptosis rate of MCF-7 in breast cancer cells was 26.1%, 51.4% and 66.4%, and the combined effect of curcumin and PD-L1 inhibitors significantly increased the apoptosis inducing effect of breast cancer cells (Fig. 3B).

3.5 Curcumin induces inhibition of PD-L1 expression and mediates inhibition of PI3K/AKT/mTOR pathway

A measurement of 5 μg/mL curcumin and 20 nM PD-L1 inhibitor BMS202 was used to act on the BC cells, and the Western blot was used to determine the level of PI3K/AKT/mTOR pathway protein expression in the breast cancer cells treated with the drug. The expression of these protein was lower than that in the blank group after the treatment with single curcumin or BMS202, and the expression level of these protein was significantly lower than that of the blank control group and the two drugs alone after the combination of the two groups (Fig. 4). The inhibitory effect of curcumin on PD-L1 induced breast cancer cell apoptosis is presumed to be related to the inhibition of the PI3K/AKT/mTOR axis.

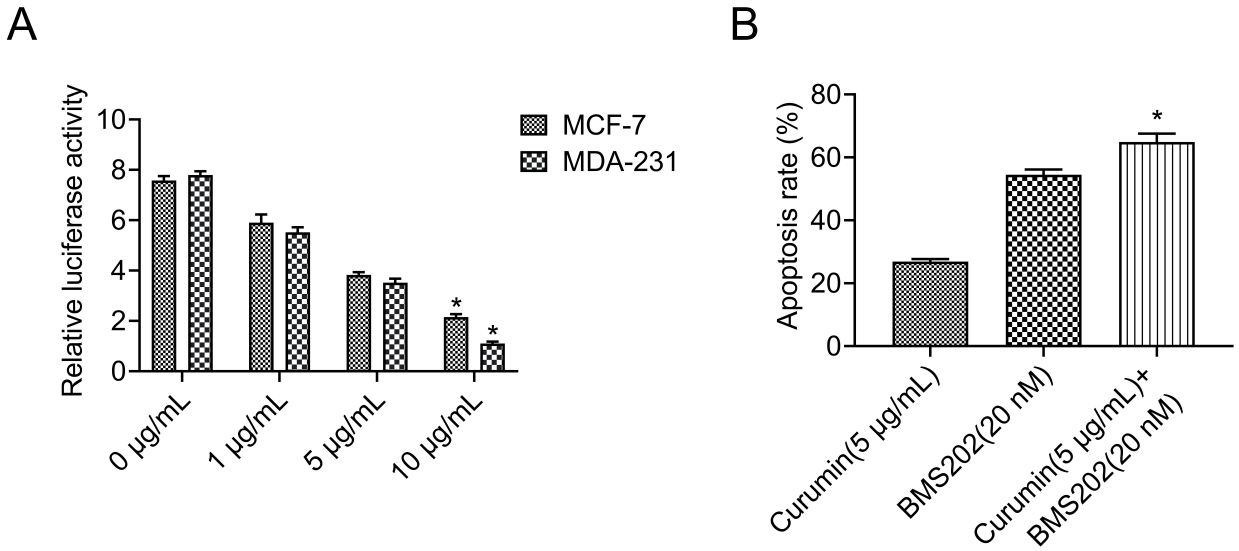

FIGURE 3. Effect of curcumin on the activity of PD-L1 promoter in breast cancer cells. (A) PGL3-PD-L1 and the internal control plasmid were co-transfected to breast cancer cells, and the activity of luciferase expression was determined by Dual-luciferase Assay System. The results showed that the activity of luciferase was significantly decreased with the increase in the concentration and the prolongation of the phase of the breast cancer cells transfected with PD-L1 promoter after treatment with curcumin. After 24 h, the activity of MCF-7 and MDA-231 decreased by 3.36 and 5.45 times respectively. (B) Curcumin combined with PD-L1 inhibitor can enhance the killing effect of breast cancer cells. 5 μg/mL curcumin and 20 nM PD-L1 inhibitor BMS202 were used to act on the breast cancer cell MCF-7, and the flow cytometry was used to determine the apoptosis level. The experimental results showed that 5 μg/mL curcumin, (20 nM) PD-L1 inhibitor BMS202 and curcumin 5 μg/mL + BMS202 (20 nM) treated for 24 h, the apoptosis rate of MCF-7 in breast cancer cells was 26.1%, 51.4%, 66.4%. *$p < 0.05$.

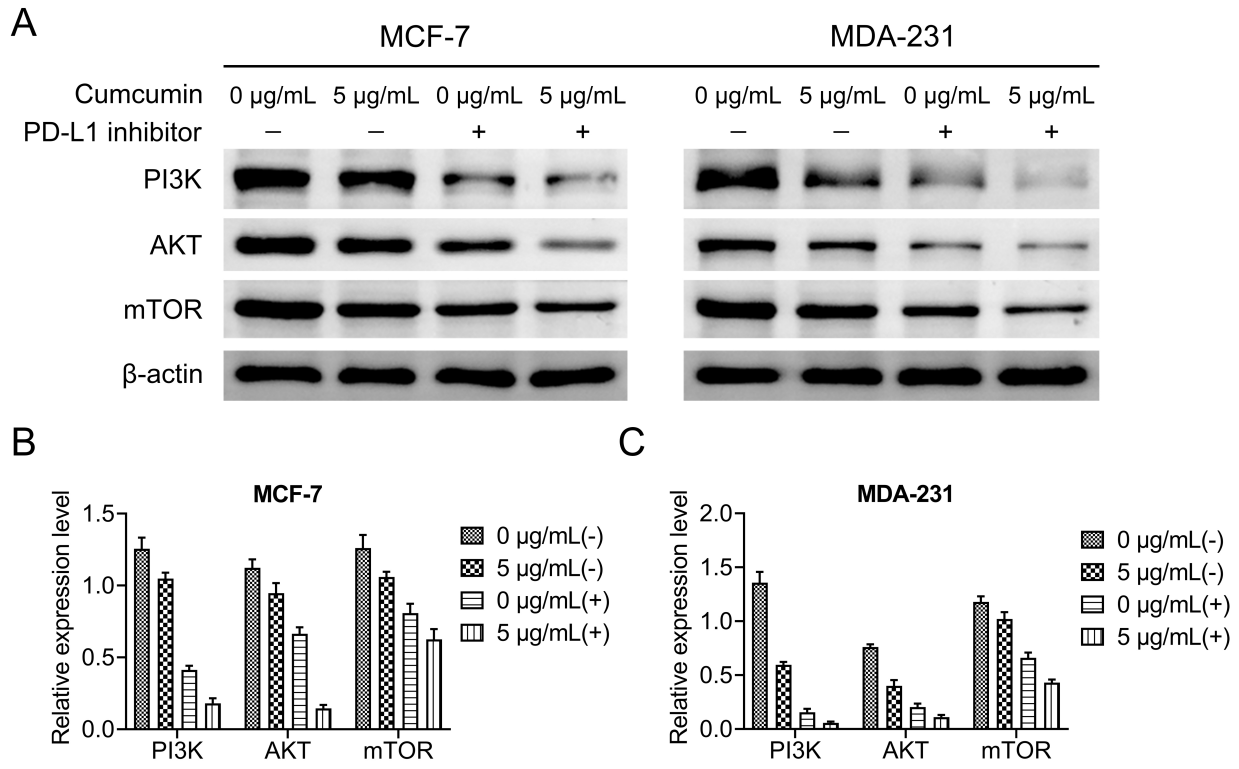

FIGURE 4. Curcumin induces the inhibition of PD-L1 expression and mediates the inhibition of the PI3K/AKT/mTOR pathway. (A) 5 μg/mL curcumin and 20 nM PD-L1 inhibitor BMS202 was used to act on the breast cancer cell MCF-7 and MDA231 cells. (B,C) The Western blot was used to determine the level of PI3K/AKT/mTOR pathway protein expression. The qualification of Western blot was used to determine the level of PI3K/AKT/mTOR pathway protein expression in MCF-7 (B) and MDA-231 (C) cells. PD-L1: Programmed Death-Ligand 1; PI3K: Phosphatidylinositol 3-Kinase; AKT: Protein Kinase B; mTOR: mechanistic Target of Rapamycin.

4. Discussion

The main pharmacological actions of curcumin include anti-inflammatory, antioxidation, hypolipidemic, anti-atherosclerosis, anti-tumor and so on, and the toxicity is low [1, 7, 8]. Curcumin is considered to be an effective antitumor drug that regulates a variety of intracellular signaling pathways, including transcription factors and receptors [9–12], kinases and growth factors [13, 14], cytokines [15], enzymes [15]. A large number of cell tests and animal experiments have proven that curcumin has definite anti-tumor activity, broad anti-tumor spectrum and little toxic and side effects, as well as is a prospect for broad spectrum application [1].

The antitumor mechanism of curcumin consists of several pathways: induces apoptosis of tumor cells [16], blocks the growth signal pathway of tumor cells [17], and inhibits the formation of tumor vessels [18]. The mechanism with which curcumin inhibits the growth of tumor cells may be [1]: inhibiting the expression of cyclin and blocking the cell cycle; influencing the expression of Smad2, Smad4 and Smad7 pathway. The apoptosis of curcumin promotes tumor cell apoptosis mainly through the following aspects: the activation of caspase-8 induces the further release of cytochrome c from mitochondria, then activates the apoptosis of the tumor cells [19, 20]. Besides, it reduces the expression of some carcinogenic transcription factors, such as NF-κB, *etc*. [10]. Also, curcumin regulates the physiological state of most tumor cells, influences angiogenesis and matrix metalloproteinase activity, and inhibits the expression of anti-chemotherapeutic factors, as well as adhesion molecules [21].

This study found that curcumin could induce apoptosis in BC cells, and the effect was inhibited by curcumin via the inhibition of the expression of PD-L1. Western blotting results showed that the expression of PD-L1 protein in curcumin-treated BC cells, decreased. To determine the expression of curcumin PD-L1 expression at the transcriptional level or in the protein level, RT-PCR was used to detect the change of the PD-L1 mRNA expression level after the gradient concentration of curcumin treatment. The results showed that the expression level of mRNA showed a decreasing trend after curcumin treatment. It seemed that curcumin inhibited the expression of PD-L1 by inhibiting transcription. The luciferase reporter gene detection test further proved that after curcumin action, the activity of luciferase was reduced by 3.36 and 5.45 times than that of the control group, and curcumin inhibited its transcription by negative regulating the promoter activity of PD-L1. This effect of curcumin also provides a new way for the treatment of BC, which inhibits the expression of PD-L1 and reverses the phenotype of malignant tumors.

In addition, curcumin has been reported to inhibit tumor invasion and metastasis [1, 8]. Fu *et al.* [22] have found that curcumin can inhibit the activity of C-Jun N terminal kinase, reduce Vascular Endothelial Growth Factor (VEGF) secretion and inhibits angiogenesis, thus achieving anti-tumor invasion and metastasis. Xu *et al.* [23] treated lung cancer cell lines with curcumin and found that curcumin could inhibit lung cancer cell migration in a dose-dependent manner. These studies suggested that curcumin had a multifaceted anti-tumor effect, indicating its considerable effect as an antineoplastic agent.

Curcumin, as a food additive, has small side effects on normal cells. The study of the combined treatment of curcumin with conventional tumor therapy has been reported repeatedly [14, 15, 21]. Chemotherapy is one of the most commonly used methods to treat cancer. Its biggest obstacle is its drug resistance. Resistance is related to NF-κB and apoptosis [24]. Curcumin significantly inhibits the activation of NF-κB induced by anticancer drugs, reduces the overexpression of P glycoprotein and apoptosis suppressor gene survivin, and reduces the activity of MMP in the invasion process of the tumor [11]. Zou *et al.* [25] explored the effect of curcumin on cisplatin sensitivity in breast cancer cells and its possible mechanism. The results showed that curcumin enhances the sensitivity of cisplatin to breast cancer cells.

Recent research has shown that curcumin can modulate immune checkpoint molecules, including PD-L1, in different cancer types, suggesting its potential as an adjunctive therapy to enhance immune responses. For example, studies in lung cancer and melanoma have demonstrated that curcumin's inhibition of PD-L1 expression can sensitize tumors to immune checkpoint inhibitors, highlighting its promise in combination therapies [2, 11]. In this study, after curcumin was found to inhibit PD-L1 expression, PD-L1 inhibitor and curcumin were combined to treat breast cancer cells simultaneously. The results suggested that the combined effects of these two drugs were significant. The inhibition and killing effect on breast cancer cells was significantly better than the single use of PD-L1 inhibitor or single curcumin.

Although our current study primarily focuses on the role of curcumin in modulating PD-L1 expression, exploring the synergistic effects of curcumin in combination with PD-L1 inhibitors on cancer cell metastasis and invasion is an important avenue for future research. Additionally, the potential of curcumin to enhance the efficacy of immunotherapies by modulating immune checkpoints like PD-L1 warrants further investigation. Future studies should aim to elucidate the molecular mechanisms underlying the interaction between curcumin and immune checkpoint inhibitors, particularly in the context of cancer metastasis and invasion. This could provide valuable insights into the therapeutic potential of curcumin as an adjunct to immune checkpoint therapy in breast cancer.

This study has some limitations. First, the experiments were conducted *in vitro*, so further *in vivo* studies are needed to confirm these findings. Second, we only focused on PD-L1, and the effects of curcumin on other immune checkpoints were not explored. Lastly, the optimal dose and treatment duration of curcumin for clinical use need to be determined through additional research.

5. Conclusions

In conclusion, curcumin demonstrates significant anti-proliferative and pro-apoptotic effects on breast cancer cell lines, with a concentration-dependent inhibition of PD-L1 expression at both the protein and mRNA levels. Additionally, curcumin enhances the apoptosis-inducing effect of PD-L1 inhibitors, suggesting its potential as a complementary agent in breast cancer treatment. Further studies are needed to validate these findings *in vivo* and explore the underlying

mechanisms in more detail.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

MJD, XYD, XJL, LZ and GZC—designed the study and carried them out; MJD, XYD, XJL, LZ—supervised the data collection; MJD, XYD, XJL, LZ—analyzed the data; MJD, XYD, XJL—interpreted the data; MJD, XYD, XJL, LZ and GZC—prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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