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Original Article

Curcumin combining with si-MALAT1 inhibits the invasion and migration of colon cancer SW480 cells

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To study the effect of small interfering RNA targeting metastasis-associated lung adenocarcinoma transcript1 (si-MALAT1) combining with curcumin on the invasion and migration abilities of human colon cancer SW480 cells, and to explore the involved molecular mechanism. The recombinant lentiviral vector expressing si-MALAT1 was constructed, and its titer was determined by gradient dilution method. The colon cancer SW480 cells with stable expression of si-MALAT1 was established, followed by treatment with curcumin at different concentrations. The effect of curcumin or si-MALAT1 alone and the combination of the two on the cell activity was detected by MTT assay. The cell invasion and migration abilities were detected by transwell and scratch-wound assay. The relative expression level of MALAT1 was detected by RT-qPCR. The protein expression was determined by Western blot analysis. The *IC50* of curcumin alone was 77.69 μmol/L, which was 51.17 mol/L when combined with curcumin and random sequence. The *IC50* of curcumin was 30.02 μmol/L when combined with si-MALAT1. The increased susceptibility multiples was 2.58. The wound healing rates were 30.9% and 67.5% after treatment with si-MALAT1 combined with curcumin for 24 hrs and 48 hrs, respectively. The numbers of invasion cells were 200±12, 162±13, 66±8, 53±4 and 16±3 after treatment with si-MALAT1 combined with curcumin for 48 hrs. The relative expression level of lncRNA-MALAT1 in the curcumin group was 68%, and the relative expression level of lncRNA-MALAT1 in si-MALAT1group was 56%, and that for the combination treatment group was about 21%. The protein expression levels of β- catenin, c-myc and cyclinD1 were significantly down-regulated upon treatment with certain concentration of si-MALAT1 alone or combined with curcumin.si-MALAT1 could significantly inhibit the invasion and migration of SW480 cells by enhancing the sensitivity of SW480 cells to curcumin. The mechanism involved mignt be related to the down-regulation of β-catenin, c-myc and cyclinD1 proteins.

Keywords: Curcumin/ pharmacology. LncRNA-MALAT1. RNA, Small Interfering/ drug effects. Colon cancer. Invasion. Migration. Colonic Neoplasms/ prevention & control. Colonic Neoplasms/ drug therapy. Neoplasm Invasiveness/ prevention & control. Cell Migration Inhibition/ drug effects.

INTRODUCTION

Colon cancer is the third most common malignant tumor in the world, and its morbidity and mortality are increasing year by year. Patient received post-operation adjuvant chemotherapy is prone to death due to metastasis and recurrence (Torre *et al.*, 2015; Burt, 2013). Therefore, it is urgent to clarify its pathogenesis and improve the therapeutic effect.

Long-chain non-coding RNA (lncRNA) is a type of non-coding RNAs with a length of more than 200 nucleotides. It is involved in many physiological and pathological processes, and considered to be closely related to the occurrence and development of tumor. They can also play roles in both tumor suppression and cancer promotion. However, the study of the molecular mechanism of lncRNA regulation is still at a preliminary stage (Fatima *et al.*, 2015). Studies have shown that the abnormal expression of lncRNA was associated with the growth and invasion of colon cancer cells (MA *et al.*, 2016). Metastasis-related lung adenocarcinoma transcript 1 (Metastasis-associated lung adenocarcinoma transcript 1, MALAT1) is widely expressed in human

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normal tissue, especially in pancreas and lung tissues (Zheng *et al.*, 2014). It is also found to be highly expressed in colon cancer tissues. Its expression and neural infiltration could affect the invasion and migration of colon cancer cells, and therefore were considered as two independent risk factors for the poor prognosis of colon cancer patients (Ji *et al.*, 2014).

Compared to the current conventional treatment strategy for cancer, traditional Chinese medicine has the advantages of low toxicity and being able to improve the patient's immunity. It will have unparalleled advantages in cancer prevention, early cancer treatment, and even in the application of middle and late stage cancer. Curcumin, a diketone compound, a substance derived from the family Curcumin. It has been shown to have anti-inflammation, anti-oxidation and anti-tumor effects, as well as reducing blood lipids (Anand *et al.*, 2008). Curcumin was also found to be able to inhibit invasion and metastasis of colon cancer cells, but the molecular mechanism involved is still not clear (Kunnumakkara, Anand, Aggarwal, 2008).

In this study, small interfering RNAs (siRNAs) were used to reduce the level of MALAT1 expression in colon cancer SW480 cells, which were further treated with curcumin.We aimed to determine the effect of this combination treatment on the viability and migration of colon cancer SW480 cells, and further clarify the potential molecular regulatory mechanism involved. It will provide the theoretical basis for the clinical treatment of colon cancer using integrated Chinese and western medicine.

MATERIAL AND METHODS

Material

Liposome LipofectamineTM2000 was purchased from Invitrogen Company; DMEM/F12 medium was purchased from Hyclone Company; RIPA cell lysate (Shanghai Sheneng betting Biotechnology Co., Ltd.); BCA protein concentration determination kit (Beijing Boosen Biotechnology Co., Ltd.). MTT reagent from the Japanese Institute of Tongren Chemistry; RNA extraction reagent TRIzol (Invitrogen Company; ECL kit (Biyuntian Biotechnology Research Institute); Polyclonal resistance to β -catenin c myc and c YclinD1 antibody, monoclonal GAPDH antibody (Cell Signaling, USA); Curcumin was purchased from Sigma Company of USA and dissolved into 10 mmol/L standby with DMSO. The recombinant lentivirus plasmid pLVX-shRNA2 and the packaging plasmid PLD2 were obtained From Shanghai Jima Co., L). And encapsulated plasmid PLD3 is provided by Shanghai Jima Co., L.

Cell culture

Colon cancer SW480 cells were purchased from Shanghai Cell Bank Center. The cells were cultured in DMEM / F12 contained 10% newborn bovine serum at 37℃ in the incubator containing 5% CO_2. Cells was digested and subcultured with 0.25% trypsin every 2-3 days. Cells with trypan blue rejection rate > 95% in logarithmic growth period were used for further experimentation.

Construction of colon cancer SW480 cells stably expressing siRNA-MALAT1

The sense chain of siRNA sequence of MALAT1 was 5'-GAGGUGUAAAGGUUUAUTT-3'. The antisense chain was 5-AUA-AAAUCCCUUU ACACCUCTT-3; The sense chain of random sequence was 5'-UUCUCCGAACGUGUC ACGUTT-3'. The antisense chain was 5-ACGUGACACGUCGGAGAATT-3; the construction of siRNA-MALAT1expressing lentiviral vector was conducted by Shanghai Jima Limited Technology Co., Ltd. Recombinant lentivirus plasmids, packaging plasmids PLD2 and encapsulated plasmids PLD3 were co-transfected into 293T cells. 24 hrs after the transfection, 10 mL fresh complete culture medium (containing 1% pairs of antibodies) was replaced. The virus-containing supernatant was then collected after 48 hrs, and then continuously diluted with a gradient of 10 times on a 48-well cell culture plate. The 10 μL of the diluted virus-containing supernatant was then added to the cell culture. After 48 hours of cultivation, the collected supernatant was diluted. The fluorescent cells were counted under inverted fluorescence microscope. The titer of the virus was defined by transducing units (TU). The virus titer was calculated according to the following formula: (Tu / m)= (green fluorescent cell number / field of vision) × (visual field number / hole number) × virus dilution multiple / virus volume . The SW480 cells were then infected with packaged lentivirus. The SW480 cell lines expressing stably siRNA-MALAT1 were established according to the instructions.

MTT assay

SW480 cells in logarithmic growth phase were cultured in serum-free DMEM/F12 medium. The cell concentration was adjusted to 1×10^5 cells / mL, and then 100 μL per well of the cell suspension inoculated in a 96-well plate. The cells were divided into two groups: blank control group (no treatment);si-MALAT1 group

(treated with 10 μmol/L of si-MALAT1); si-MALAT1 combined with curcumin group (treated with 15μmol/L of si-MALAT1 and curcumin); Negative control group (treating with 15μmol / L of random sequence) and the Curcumin group (treated with 5, 10,20,40 and 80 μmol/L of curcumin); after 48 hrs of cultivation, 20μL of MTT solution was added to each well. After 4 hrs, the culture medium was carefully discarded, and 150 μL DMSO was added to each well. The resultant violet crystal was completely dissolved by oscillating at low speed for 10 min. The absorbance of each well was measured at the wavelength of 490 nm and the average value was obtained.

Transwell assay

The Matrigel was diluted in serum-free DMEM/F12 medium and then added into the transwell chamber. containing 8 μm small polycarbonate filter membrane. 5×10^5 of SW480 cells were diluted in 100μL serum-free medium, and 600μL of the DMEM/F12 medium containing 10% serum was added in the lower chamber. Each group was cultured for 24 hrs in triplicate at 37℃ in the incubator containing 5% CO_2. Cells in the culture chamber was then removed and fixed with methanol for 20 mins, followed by staining with 1% crystal violet for 20 mins. The matrix glue and the non-invasive cells at the bottom of the upper chamber were gently wiped off with wet cotton swabs, then the chamber was inverted. The number of cells passing through the membrane was counted under the 100x optical microscope.

Scratch-wound assay

5×10^5 of SW480 cells were added to each well of a 12-well plate and allowed to grow until a confluent monolayer was formed. Then the cell monolayer was scraped in a straight line to create a gap with a pipet tip. The cell debris was then removed by washing with 1xPBS for 3 times. Culture medium was then added again and cells were allowed to grow for 48hrs before taking the microscopy imagines. The distance between one side of scratch and the other was measured using Image J software.

Detection of relative expression level of MALAT1 by RT-qPCR

M-MLV Reverse Transcriptase was used for the reverse transcription of mRNA to complementary DNA (cDNA), which was later used as the PCR template. Each experiment was performed in triplicate. The expression levels of U6 and U6 was used as an internal control for mRNAs. The sense chain sequence of primer. MALAT1 was 5'-AGGCGTTGTGGTAGAGA-3'. The sequence of antisense chain is 5 GGATTTACCAACCACTCGC-3; The sense chain sequence of U6 was 5'-CTCGCTT CGGCAGCACA3'. The antisense sequence was 5 AACGCTTCACGAATTTGCGT-3. Using U6 as internal reference, the relative expression of MALAT1 was calculated.

Western blot analysis

The cell was lysed in RIPA buffer containing a proteinase inhibitor cocktail (Biocolor BioScience & Technology, Shanghai, China). After centrifugation at 4 °C for 15 mins, the pellets were discarded. The protein concentration of each group was determined by bicinchoninic acid (BCA) Protein Assay Kit (Bioss, Beijing, China). The protein was then separated on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF membranes. GAPDH was used as the internal reference. The ratios of β-catenin,c-myc,cyclinD and GAPDH were calculated and interpreted as the relative expressions of β-catenin,c-myc and cyclinD1 proteins. All experiments were repeated for three times (absolute gray value = gray value - background gray value).

Statistical analysis

All the results were represented the average of at least three independent experiments. Data were expressed as the mean ± SD and analyzed by one-way analysis of variance (ANOVA) and Student's *t* test. A $p<0.05$ was considered statistically significant.

RESULTS

The lentiviral vector expressing si-MALAT1 was successfully constructed

The recombinant lentiviral plasmid, packaging plasmid PLD2 and the envelope plasmid PLD3 were co-transfected into 293T cells. The virus-containning supernatant was then collected at 72 h, which viral titer was 1x109 TU/mL determined using 10 times continuous gradient method(Figure 1A). The GFP virus expression was observed by fluorescence microscope (Figure 1B). Sequencing results of the recombinant plasmid products were completely compatible with the designed primer sequences using the Blast ratio. It suggested that the

lentivirus expression vector was successfully constructed (Figure 1C).

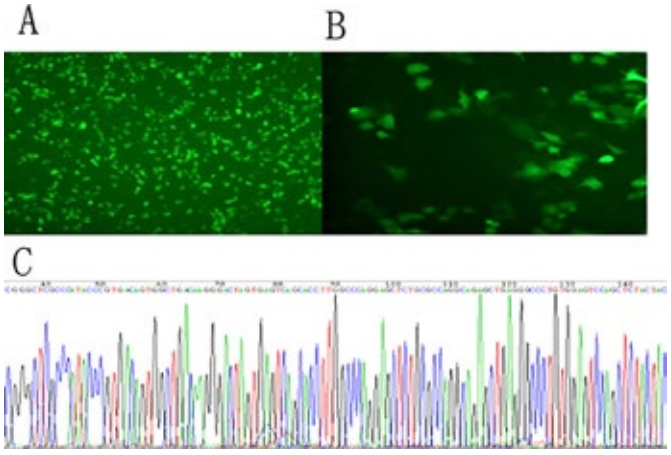

FIGURE 1 - Determination of lentivirus titer and identification of recombinant plasmid products by sequencing. A: The recombinant plasmid expressing si-MALAT1 was observed under fluorescence microscope (40x); B: The plasmid expressing empty vector was observed under fluorescence microscope; C: Identification of recombinant plasmid products by sequencing .

Curcumin combined with si-MALAT1 inhibited the SW480 cell viability

The *IC50* of curcumin alone was 77.69 umol·L^{-1}. The *IC50* of curcumin combined with random sequence was 51.17 umol·L^{-1}; The *IC50* of curcumin combined with si-MALAT1 mimics was 30.02 umol·L^{-1}. The results showed that curcumin combined with si-MALAT1 could significantly inhibit the viability of SW480 cells (Figure 2).

Curcumin combined with si-MALAT1 inhibited the migration of SW480 cell

SW480 cells were treated with certain concentration of si-MALAT1 mimics, random sequences, curcumin and si-MALAT1mimics on for 24 h or 48 h. The wound healing rate of the curcumin group alone was 30.9% and 67.5% after treatment for 24 h and 48 h, respectively. The wound healing rate of si-MALAT1 mimics combined with curcumin group was 15.75% and 24.8% after treatment for 24 h and 48 h, respectively. The si-MALAT1 analogue combined with curcumin group had significantly lower wound healing rate than those of the blank control group and the negative control group ($P < 0.05$).There was no significant difference between the negative control group and the blank control group ($P > 0.05$).The results suggested that Si-MALAT1 combined with curcumin could significantly inhibit the migration of SW480 cells. (Figures 3A and B)

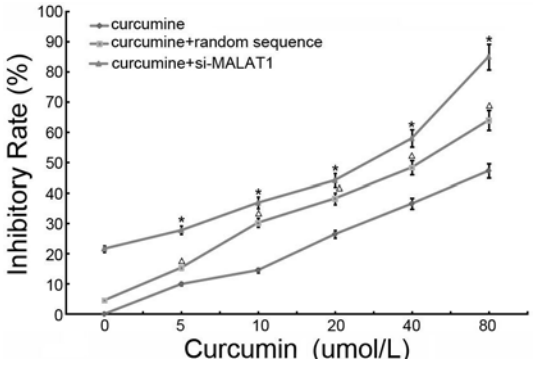

FIGURE 2 - Cell inhibition rates in the curcumin group, the curcumin and random sequence group, and the curcumin and si-MALAT1 group *P<0.05, $^{\triangle}P$> 0.05, n=3.

Curcumin combined with si-MALAT1 inhibited the invasion of SW480 cell

Result from the transwell assay showed that, the number of invasion cells for the blank control group, the negative control group, the curcumin group, and the curcumin combined with si-MALAT1 mimics group were 200±12,162±13,66±8,53±4and16±3, respectively. The number of invasion cells in the curcumin combined with si-MALAT1 mimics group was significantly lower than those in other groups ($P < 0.05$). It suggested that si-MALAT1 combined with curcumin could inhibit the invasion of SW480 cells (Figure 4).

Curcumin combined with si-MALAT1 inhibited the expression of MALAT1

SW480 cells were treated with curcumin alone or combined with si-MATAT 1 at a certain concentration for 48 hrs, and the expression of lncRNA-MALAT1 was then detected by real-time quantitative PCR. The results showed that the relative expression levels of lncRNA-MALAT1 were 68%, 56% and 21% in the curcumin group, the si-MALAT1 group, and the combination treatment group, respectively. The difference between the combination treatment group and the single treatment group was statistically significant ($P < 0.05$) (Figure 5).

Curcumin combined with si-MALAT1 down-regulated the protein expression of c-myc, cyclinD1 and β-catenin

si-MALAT1 was transfected into SW480 cells for 48 h. The results of Western blotting showed that the

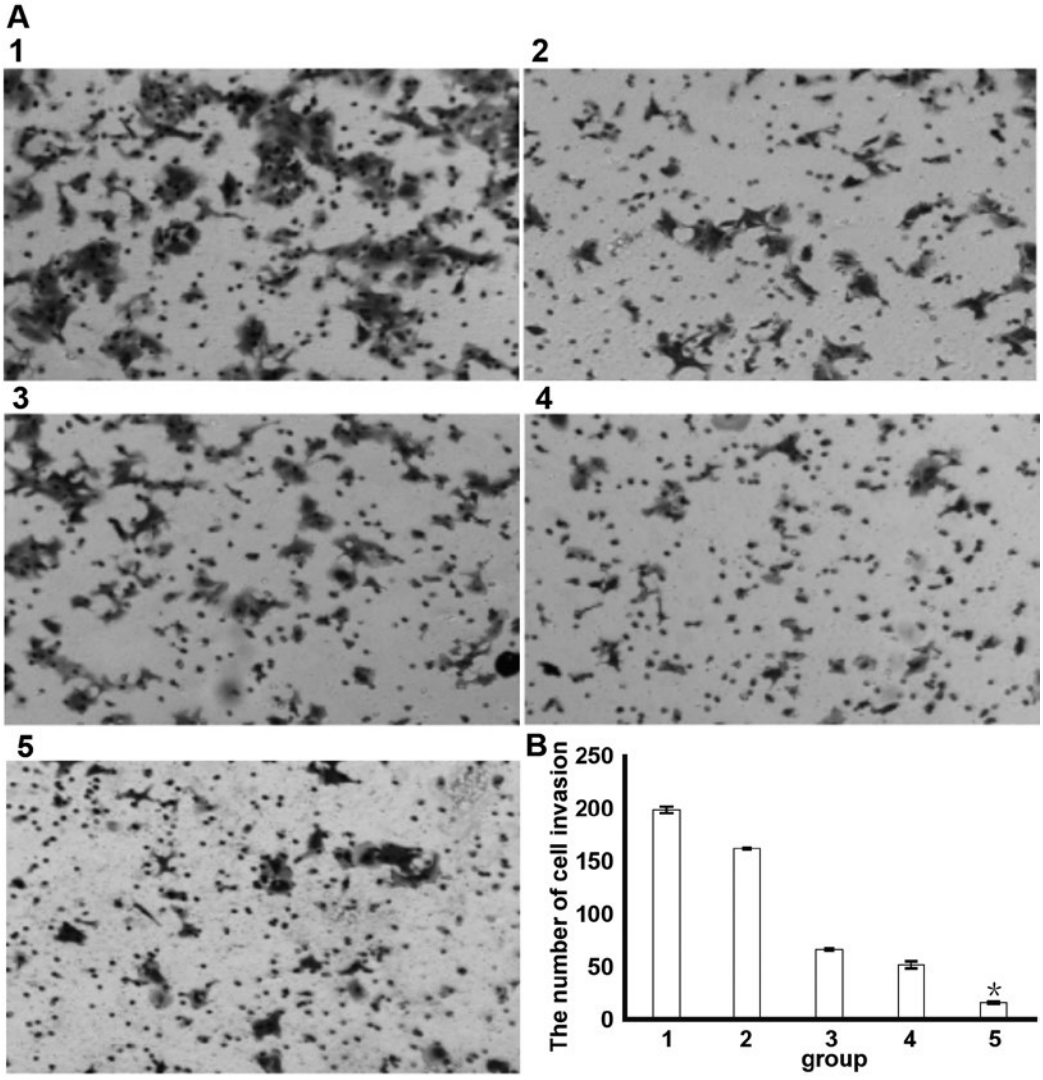

FIGURE 3 - The migration ability of SW480 cells was detected by transwell assay. A: The cell migration distance of each group after treatment for 24 h or 48 h was observed under fluorescence microscope (40X); C: The quantitative analysis of of the wound healing rates in each group after treatment for 48hrs. Mean±SD. n= 3. * P<0.05, * * P<0.05.

protein expression levels of β-catenin, c-myc and cyclinD1 were significantly down-regulated when treatment with certain concentration of si-MALAT1 mimics alone or combined with curcumin (Figure 6).

DISCUSSION

Colon cancer is one of the most common malignant tumors of digestive tract in the world, which is seriously threatening human life and health. The occurrence and development of colon cancer is a multi-step process. The mortality rate of colon cancer in China is as high as 60%. The conventional treatment for early colorectal cancer is surgical resection. The 5-year survival rate can reach 90% to 95%. The metastasis and invasion of colon cancer are the main causes of postoperative recurrence and the death of patients (Wu *et al.*, 2014). Therefore, clarifying the molecular mechanism involved in the invasion and metastasis of colon cancer cells will shed the light on the development of effective therapeutic treatment strategy for colon cancer, and hopefully lower its mortality rate.

The differential expression of lncRNA in colon cancer is closely related to clinical features, suggesting that lncRNA is involved in the invasion and metastasis of colon cancer (Chen *et al.*, 2016). Some studies have shown that lncRNA-MALAT1 was highly expressed in colon cancer tissues, and high expression of lncRNA-MALAT1 was associated with poor prognosis and lower chemotherapeutic tolerance (Mercer, Dinger, Mattick, 2009). Curcumin is a type of polyphenols extracted from the rhizomes of turmeric and other plants. It has a wide range of pharmacological usage. It has been shown that

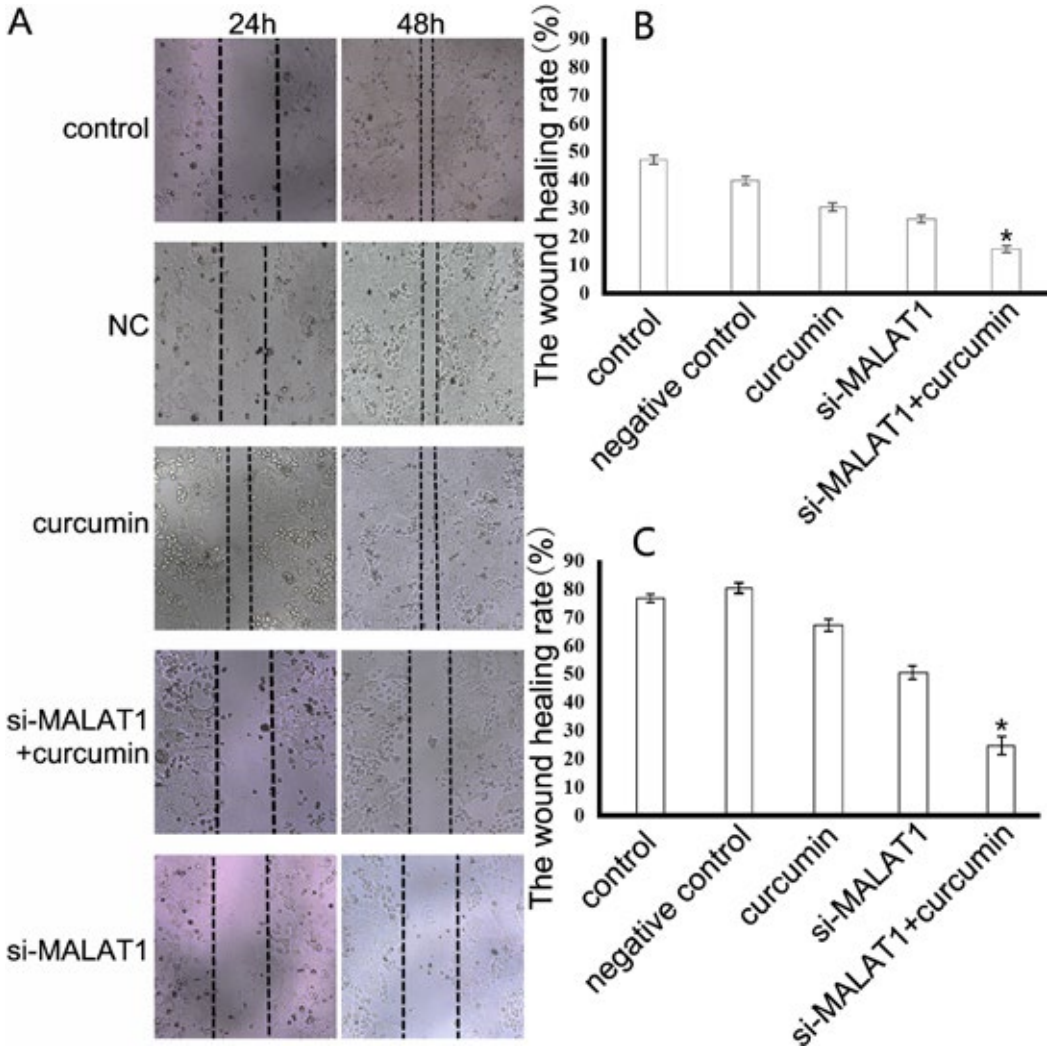

FIGURE 4 - The invasion ability of SW480 cells in different groups was evaluated by scratch-wound assay. A: The invasion cells in each group were observed under fluorescence microscope (40X; B: The quantitative analysis of the invasion cells in each group: 1: Control group; 2: Negative control group; 3: Curcumin group; 4: si-MALAT1 group; 5: Curcumin combined with si-MALAT1 group. Mean±SD. n=3 . * P<0.05 *vs* other groups.

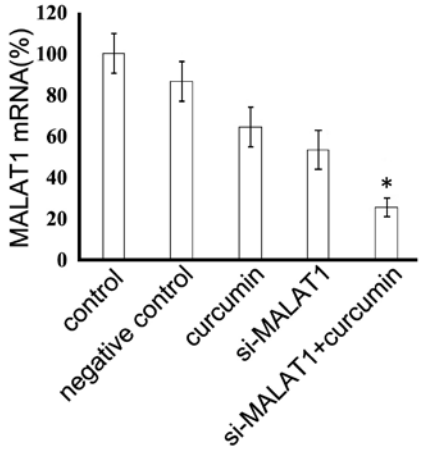

FIGURE 5 - The effect of curcumin on the expression of lncRNA-MALAT1 in the human colon cancer SW480 cells. Mean±SD. n=3 . * P<0.05 *vs* other groups.

curcumin could inhibit metastasis and induce apoptosis in many kinds of tumors (Zhou *et al.*, 2014).

In this study, the small interfering RNA sequence for MALAT1 was designed, and the recombinant lentivirus plasmid pLVX-shRNA2 was ligated with the encapsulated plasmid. The recombinant lentivirus plasmid si-MALAT1 was constructed, and used for infecting SW480 cells to establish a stable cell line expressing si-MALAT1. At the same time, the combination of si-MALAT1 with different concentrations of curcumin could significantly increase the sensitivity of SW480 cells to curcumin after treatment for 48 hrs. Curcumin combined with si-MALAT1 could inhibit migration and invasion of SW480 cells.

Wnt signaling pathway is not only closely related to embryonic development, but also contributes to human tumorigenesis (Heintze *et al.*, 2017). β-catenin avoids

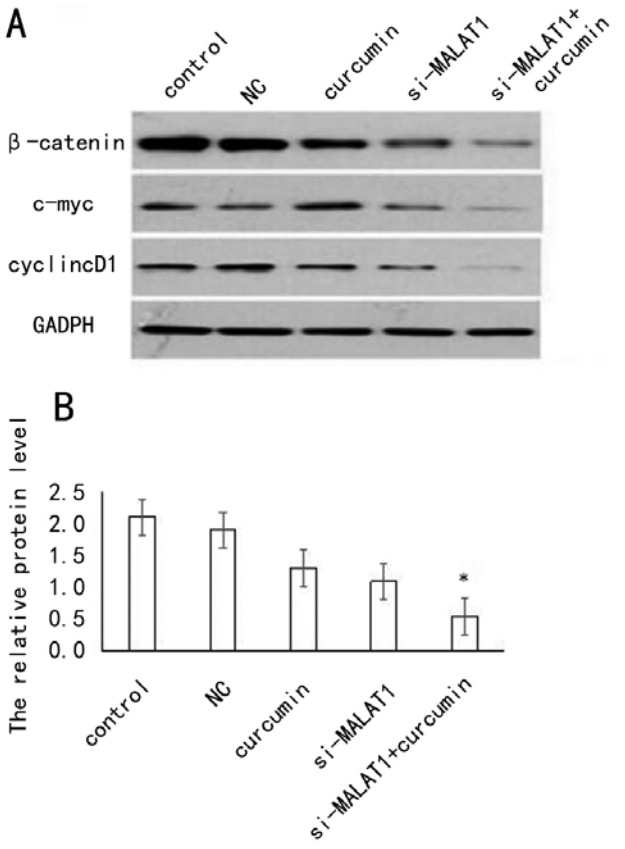

FIGURE 6 - The relative protein expression levels of β-catenin, c-myc and cyclinD1 detected by Western-blotting. A:β-catenin, c-myc and cyclinD1 expression in different treatment groups; B:.The relative protein expression levels of catenin, c-myc and cyclinD1 in different treatment groups, Mean±SD. n=3 .* P<0.05 *vs* other groups.

degradation through ubiquitin proteasome pathway and enters nucleus aggregation to activate downstream target genes such as c-myc. Expression of cyclin D1 and VEGF can promote tumor occurrence and metastasis (Bienz, Clevers, 2000). In this study, 48 hrs after treatment with curcumin and si-MALAT1, the expression of MALAT1 in SW480 cells was down-regulated. At the same time, the protein expression of cyclin D1, β-catenin and c-myc was also decreased. The results suggested that curcumin combined with si-MALAT1 could inhibit the proliferation of SW480 cells. Migration and invasion of SW480 cells were achieved by down-regulating MALAT1 and down-regulating cyclin D1.

β-catenin and c-myc in the Wnt signaling pathway .Curcumin could obviously suppress the growth of SW620 in colon cancer cells *in vitro* and *in vivo* through inhibiting the Wnt signaling pathway by up-regulating the expression of NKD2 gene, and then, down regulating expression of CXCR4 in tumor cells, which further inhibited the invasion and metastasis of tumor cells (Akira *et al.,* 2000).

CONCLUSIONS

In conclusion, this study demonstrated in vitro that, curcumin combined with si-MALAT1 inhibited the viability, migration and invasion of SW480 cells by down-regulating the expression MALAT1. And down-regulating the expression of β -catenin, c-myc and cyclin D1 in Wnt signaling pathway. However, the *in vivo* effect of curcumin needs to be further elucidated. The present study provides the theoretical basis for the clinical treatment of colon cancer using integrated Chinese and western medicine, and will hopefully aid the development of new therapeutic treatment strategy for colon cancer in the near future.

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