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Protective Effects of Curcumin on Colon Cancer Based on Regulation of lncRNA NBR2 Targeting Notch1 for Epithelial-mesenchymal Transition

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KEYWORDS Colon Cancer. Curcumin. Epithelial-Mesenchymal Transition. Notch1. Proliferation

ABSTRACT The researchers set the purpose of appraising how curcumin performs its protective function in colon cancer based on regulation of lncRNA NBR2 targeting Notch1 for epithelial-mesenchymal transition. Curcumin at 10-50 μmol/L inhibited SW480 cell proliferation. With rising curcumin concentration, the protein expression of Vimentin significantly decreased, whereas E-cadherin presented an increased protein expression ($P<0.05$). Curcumin up-regulated NBR2 expression but down-regulated Notch1 expression dose-dependently. NBR2 and Notch1 expressions were negatively correlated with each other. The Notch1 expression in curcumin treatment groups was suppressed by NBR2 overexpression, but enhanced by NBR2+Notch1 treatment. The NBR2+Notch1 group exhibited a strengthen cell proliferation ability compared with the NBR2 group. In curcumin treatment groups, the roles of NBR2 overexpression in suppressing cell proliferation was weakened by overexpressed Notch1. LncRNA NBR2 is a vital player in regulating curcumin to exert its inhibitory effects on cell proliferation, probably by down-regulating the Notch1 pathway in the case of colon cancer.

INTRODUCTION

Colon cancer originates from malignantly transformed colonic epithelial cells, which is the third most common carcinoma worldwide (Na et al. 2020). Due to high morbidity and mortality rates, it has posed severe threats to the health of the public. The pathogenesis of colon cancer remains unclear, and it is mostly derived from adenomatous polyps and affected by diet, genetics, age, mental factors, chemical carcinogens, digestive tract diseases and unhealthy living habits (Malki et al. 2020). The common symptoms of colon cancer patients are weight loss and emaciation caused by malnutrition due to digestive function decline, and left- and right-sided colon cancer is mainly manifested as hemafecia, diarrhoea, abdominal pain, loss of appetite, fatigue and vomiting (Yang et al. 2019). Despite advanced medical treatments such as surgical resection, chemotherapy, radiotherapy and targeted therapy, the recurrence and metastasis of colon cancer are still the main causes for death (Li et al. 2019).

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Curcumin exerts inhibitory effects on diversified tumours, ranging from lung cancer, colon carcinoma to breast carcinoma (Giordano and Tommonaro 2019). It may suppress the proliferation of colon cancer cells through regulating the Notch1 signalling pathway, as a new strategy for treating colon cancer (Wang et al. 2019). Usually belonging to RNA transcripts lengthening over 200 nt and lacking the effective protein-coding ability, long non-coding ribonucleic acids (lncRNAs) participate in tumorigenesis through transcriptional and post-transcriptional gene regulation (Zhu et al. 2021). LncRNAs often work as oncogenes or tumour suppressor genes, and their dysregulation has intimate associations with tumour onset (Tian et al. 2018). Besides, many lncRNAs can regulate carcinoma cells from the aspects of metastasis and propagation (Chen et al. 2018). The onset and progression of colon cancer depend not only on the cancer cells themselves, but also on the microenvironment and regulation of inflammatory tumours (Zhong et al. 2019). Notch1, a Notch receptor, is implicated in regulating tissue and cell proliferation and apoptosis as a transmembrane protein, and expressed in many tumours (Gharaibeh et al. 2020). It is also

one of the target genes of NBR2 (Morshedi et al. 2021). Present in epithelium-derived tumours including breast carcinoma, esophageal carcinoma as well as colorectal carcinoma, epithelial-mesenchymal transition (EMT) has close correlations with tumour invasion and metastasis (Çelebier et al. 2019). Xue (2013) reported that down-regulating the expression of Notch1 inhibited EMT in esophageal squamous carcinoma cells, thereby suppressing their proliferation. In addition, Cai et al. (2019) found that NBR2 exerted antitumor effects on osteosarcoma through regulating Notch1 signal transduction and EMT. Currently, the influence of curcumin on NBR2 targeting Notch1 in colon cancer cells has seldom been reported.

Objectives

The expressions of NBR2 and Notch1 in colon cancer cells were detected, and the mechanism by which NBR2 targeted Notch1 to regulate the inhibitory effects of curcumin on colon cancer cell proliferation was explored, aiming at establishing foundations of theory for treatment in clinic.

METHODOLOGY

Materials and Apparatus

Shanghai Cell Bank of Chinese Academy of Sciences (China) was the supplier of human colon adenocarcinoma cell line (SW480). Dulbecco's modified Eagle medium (DMEM) (Gibco, USA), kits for quantitative real-time polymerase chain reaction (qRT-PCR) (UNIBIOTEST, Wuhan, China), curcumin (Sigma, USA), methyl thiazolyl tetrazolium (MTT) assay kits (Sigma, USA), protein A/G coupled sepharose (Santa Cruz, USA), GAPDH monoclonal antibody, goat anti-rabbit secondary antibody plus polyclonal antibody against β-actin (KeyGEN, China) and bicinchoninic acid (BCA) protein concentration assay kits (Rebstock, Germany) were used. Shanghai Genechem Co. Ltd. (China) was responsible for synthesizing and packaging luciferase reporter vector NBR2 and Notch1 overexpression lentivirus, while the primers of EMT-related epithelial and mesenchymal marker molecules were synthesised by Zhejiang Hangzhou LC-Bio Co. Ltd. (China).

The following instruments were employed: super clean bench (Beijing Guanpeng Purification Equipment Co. Ltd. China), cryogenic refrigerator (-80°C, Wiggens, Germany), high-speed low-temperature centrifuge (Beijing Liuyi Instrument Factory, China), gel imaging system (Bio-Rad, USA), and flow cytometer (Thermo, USA).

Curcumin for Cell Treatment and Culture

An incubator containing DMEM supplemented with 100 μg/mL streptomycin, 10 percent fetal bovine serum, plus 100 μ/mL penicillin was employed to culture SW480 cells under the conditions of 37°C, 5% CO_2 and full humidity. Then trypsin digestion together with 6-well plate inoculation (1.5×10^5/well) was performed for the cultured cells in the logarithmic growth phase. When the cells covered about 70-80 percent of the plate wall, they were treated with curcumin at different content levels (0 μmol/L, control group; 10, 20, 30, 40 and 50 μmol/L) for 6, 12, 24 and 48 h, followed by proliferation detection.

Detection of Cell Proliferation

A plate with 96 wells was used for SW480 cell inoculation and overnight culture to promote the adherence. After curcumin at different concentrations was added, MTT assay was conducted at different time points to determine the cell viability, with untreated cells as negative control. After the addition of MTT (5 mg/mL, 20 μL) into every well, 4 hours of cell culture in the incubator was carried out, and then the solution was discarded to terminate the reaction. The bluish-purple precipitate was dissolved with 150 μL of DMSO in each well for 10-minute low-speed shaking, and 490 nm was selected to measure the absorbance. With the result of the untreated group as 0.00 percent, the inhibition rate was calculated. The assay was repeated 3 times to ensure reliability.

qRT-PCR

The expressions of NBR2 and Notch1 were determined by RT-PCR. The TRIzol method was adopted to extracted total RNA, while RT-PCR kit was utilized for cDNA synthesis, followed by qRT-PCR. The primer sequences were as follows. NBR2: F-5'-GGAGGTCTCCAGTTTCGGTA-3', R-5'-

TTGATGTGTGCTTCCTGGG-3', Vimentin: F-5'-GAGTCCACTGAGTACCGGAGAC-3', R-5'-TGTAGGTGGCAATCTCAATGTC-3', Notch1: F-5'-GAGGCGTGGCAGACTATGC-3', R-5'-CTTGTACTCCGTCAGCGTGA-3', and E-cadherin: F-5'ACCTTGTGCCGCGTAAGACAG-3', R-5'-CGTCAGCGTCAGTGTCAGGAA-3'. The reaction process included totally 40 cycles of 3 minutes of 75°C pre-denaturation, 5 minutes of 95°C denaturation, 30 seconds of 60°C annealing, and 30 seconds of 72°C extension. The relative expression was expressed as $2^{-\Delta\Delta CT}$.

Cell Transfection

SW480 cells seeded in a plate with 6 wells (10^5/well) were cultured with DMEM for 24 hours, and washed with serum-free medium. Transient transfection plus RNA overexpressing NBR2, overexpressing NBR2+Notch1 and NC was separately performed for the cells *as per* the instructions of kits. At 24 hours after transfection, 30 ìmol/L curcumin was added for 36 hours of cell treatment, with cells collected for further analysis. The grouping of cells involved Control group (not transfected), NC group (transfected with lentiviral vector Lv-mmu), NBR2 group (transfected with Lv-mmu NBR2 overexpression lentivirus), and NBR2+Notch1 group (transfected with Lv-mmu NBR2 overexpression lentivirus and Lv-mmu Notch1 overexpression lentivirus).

Bioinformatics Prediction

Whether lncRNA NBR2 interacted with Notch1 was predicted through the official website of RNAInter (http://www.rna-society.org/raid/search.html). Besides, the correlation between them was visualised by a network diagram.

Coimmunoprecipitation Assay

SW480 cells overexpressing Notch1 were chosen for extraction of total protein, whose concentration was determined by spectrophotometry. Before use, a -80°C refrigerator was employed to preserve 15 μg of proteins. The remaining proteins were equally divided into two groups and added about 2 μg of Notch1 antibody and control IgG, followed by overnight (4°C) incubation. Then protein A/G-coupled Sepharose (20 μL) was added for 6-hour reaction, which were subjected to 3 minutes of 3,000 rpm centrifugation. Then collection of co-immunoprecipitation complex was implemented together with washing with phosphate buffered saline containing protease inhibitor PMSF 3 times. The stored protein and the above two groups of proteins were added 20 μL of 2× protein loading buffer, subjected to boiling water bath for 10 minutes and centrifuged. The supernatant was collected for Western blotting.

Detection of EMT Marker Expressions by Western Blotting

SW480 cells were collected, added 500 μL of protein lysis buffer blended with protease inhibitor (5 μL) and phosphatase inhibitor (5 μL) in each well, and subjected to 20 minutes of shaking and lysis. Afterward, the cells underwent 30-minute 4°C centrifugation with a speed of 12,000 rpm. Total protein was extracted from the tissue homogenate using an extraction kit and quantified with BCA according to the instructions. The BCA kit was applied to determine protein concentration. After denaturation, each well of proteins (30 μg) received SDS-PAGE and membrane transfer, and the primary and secondary antibodies were added for blocking and incubation. Finally, the membrane was exposed, and protein expressions were analysed using the image analysis software, during which the internal reference was determined as GAPDH.

Statistical Analysis

Statistical analysis was accomplished by virtue of SPSS 25.0 software, while plotting was executed *via* GraphPad 5.0 software. The intergroup comparison was realized by means of *t* test. Differences of statistical significance were denoted as $P<0.05$.

RESULTS

Effects of Curcumin on Colon Cancer Cell Proliferation

Curcumin at different content levels (10, 20, 30, 40 and 50 μmol/L) was used for SW480 cell treatment. Later, MTT assay was implemented at different time points to examine the cell viability.

Compared with the Control group, SW480 cells were inhibited by curcumin at 10-50 μmol/L to different extents, and the inhibition rate rose with increasing concentration (P<0.05). As shown in Figure 1, the inhibition rate also gradually rose with prolonged time, especially at 12, 24 and 48 hours.

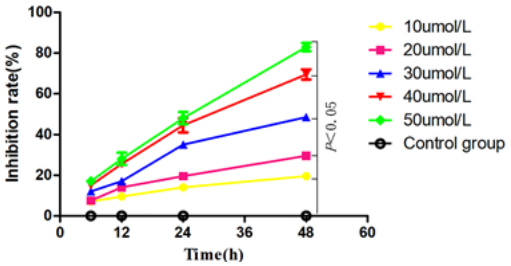

Fig. 1. Inhibition rates of curcumin at different concentrations on colon cancer cells detected by MTT assay

Functions of Curcumin in affecting EMT-related Proteins in Cells of Colon Carcinoma

Western blotting was adopted to investigate the role of curcumin in impacting the proteins associated with EMT (Vimentin and E-cadherin) in colon cancer cells. With the internal control set as β-actin, the changes in E-cadherin and Vimentin levels showed opposite trends. In contrast to that in the Control group, Vimentin was down-regulated at the protein expression level after curcumin treatment depending on the concentration (P<0.05). After curcumin treatment, concentration-dependent up-regulation of E-cadherin protein expression was observed (P<0.05) (Fig. 2).

Role of Curcumin in influencing NBR2 plus Notch1 Expressions in Colon Cancer Cells

After treatment with curcumin at different concentrations for 24 hours, SW480 cells were collected, in which qRT-PCR was performed to measure NBR2 plus Notch1 expressions. The expression of Notch1 was dose-dependently inhibited by 10-50 μmol/L curcumin in comparison to that in the Control group (P<0.05). Additionally, NBR2 rose at the expression level in curcumin treatment groups concentration-dependently (P<0.05). Meanwhile, according to Figure 3, a negative relationship between NBR2 and Notch1 expressions was observed (Pearson *r*=0.764, P<0.05).

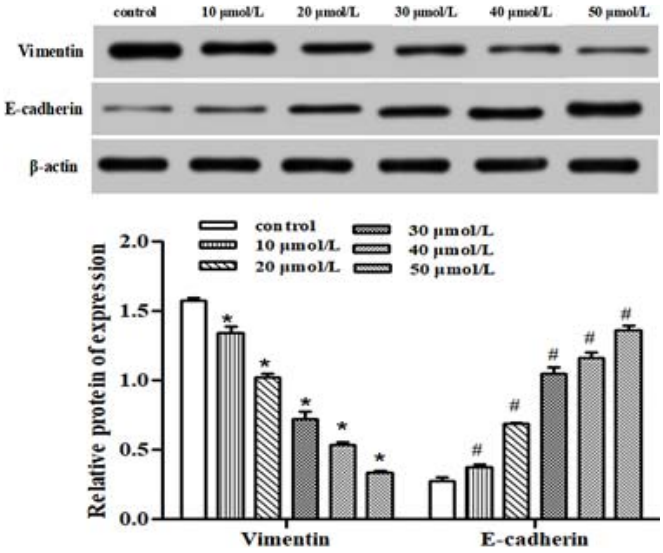

Fig. 2. Effects of curcumin at different concentrations on expressions of EMT-related proteins in SW480 cells. A: Protein expression detected by Western blotting; B: relative expression level. *P<0.05 (Vimentin protein level) and #P<0.05 (E-cadherin protein level) vs. Control group

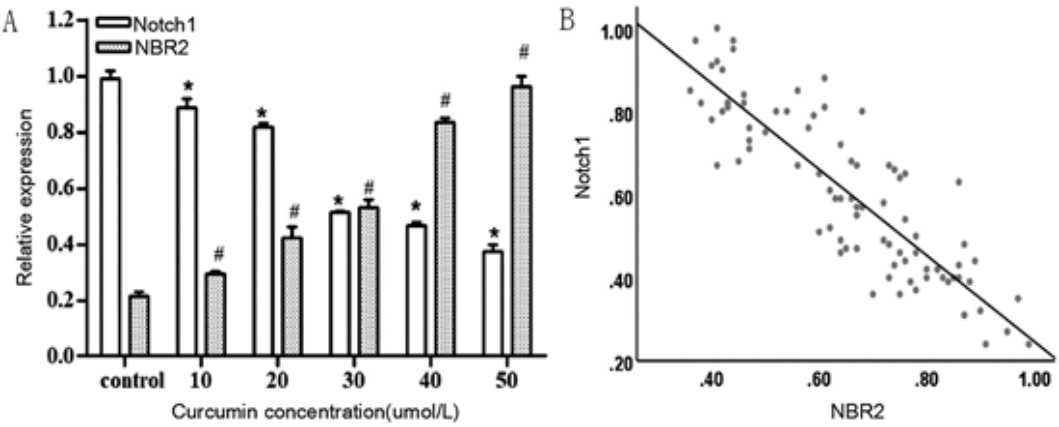

Fig. 3. Effects of curcumin at different concentrations on expressions of NBR2 and Notch1 in SW480 cells. A: Expression levels of NBR2 and Notch1 in SW480 cells treated with curcumin at different concentrations; B: analysis of correlation between NBR2 and Notch1 expressions

7Interaction between NBR2 and Notch1 Studied by Coimmunoprecipitation Assay

Whether NBR2 interacted with Notch1 was predicted through the RNAInter website. There was interaction between NBR2 and Notch1 (Fig. 4A). Besides, whether there was physical binding between NBR2 and Notch1 was detected by co-immunoprecipitation assay, using GAPDH as the internal reference. NBR2 was able to precipitate Notch1, verifying that there was physical binding between NBR2 and Notch1 in SW480 cells (Fig. 4B).

Effects of NBR2 Targeting on Notch1 Expression and Cell Proliferation

The effects of NBR2 overexpression, and NBR2 overexpression + Notch1 overexpression on the biological activity of SW480 cells treated with curcumin were assessed. The NBR2 group had an inhibited expression of Notch1 by comparison with the NC group, but by contrast to the NBR2 group, the NBR2+Notch1 group exhibited an up-regulated Notch1 expression ($P<0.05$) (Fig. 5A). The MTT assay results revealed that the NBR2 group possessed weaker SW480 cell pro-

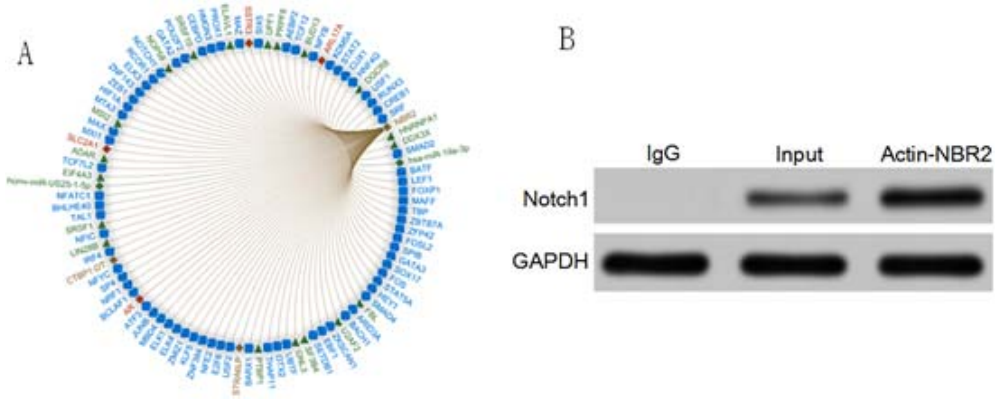

Fig. 4. Interaction between NBR2 and Notch1. A: Interaction between NBR2 and Notch1 in RNAInter database; B: interaction between NBR2 and Notch1 detected by coimmunoprecipitation assay

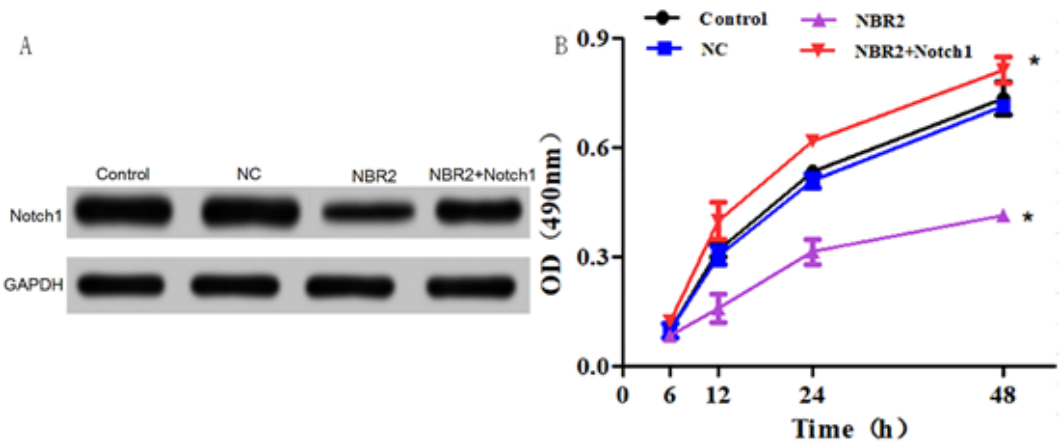

Fig. 5. Effects of NBR2 targeting Notch1 on Notch1 expression and cell proliferation. A: Notch1 protein expression detected by Western blotting; B: effect of NBR2 targeting Notch1 on cell proliferation

liferation than the NC group, whereas compared to that in the NBR2 group, the NBR2+Notch1 group displayed enhanced SW480 cell proliferation ($P<0.05$). Taken together, the overexpression of Notch1 reversed the function of NBR2 overexpression in suppressing Notch1, enhancing colon carcinoma from the perspective of cell proliferation (Fig. 5B).

Effects of NBR2 Targeting Notch1 on EMT-related Protein Expressions in Cells

To explore the role of NBR2 targeting Notch1 in affecting the protein expressions correlated with EMT in colon cancer cells, the changes in such proteins were explored through Western blotting. There were no significant changes in the Control or NC group. The levels of N-cadherin, Notch1, and Vimentin declined, whereas E-cadherin level rose in the NBR2 group compared with the NC group. However, the changing trends of the levels of Notch1, Vimentin, N-cadherin and E-cadherin between the NBR2+Notch1 group and the NBR2 group were opposite to those between the NBR2 group and the NC group. Collectively, the overexpression of Notch1 attenuated the down-regulation of NBR2 overexpression on Notch1 in curcumin treatment groups, that is, overexpressing Notch1 reversed the inhibitory effect of NBR2 on cell proliferation (Fig. 6).

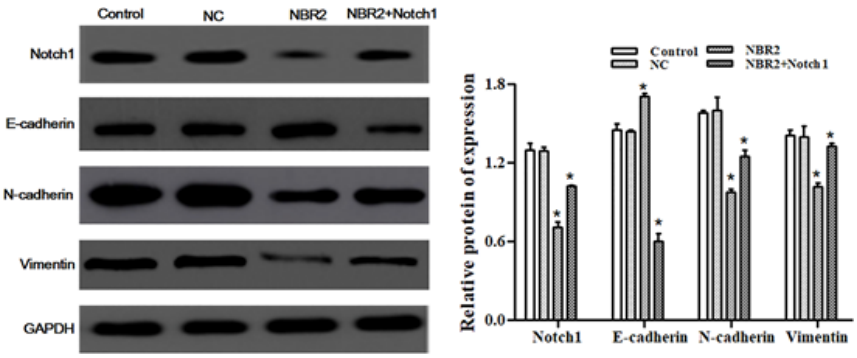

Fig. 6. Effects of NBR2 targeting Notch1 on expressions of EMT-related proteins. *$P<0.05$ vs. NC group, *$P<0.05$ vs. NBR2 group

DISCUSSION

Being a major cause for tumour-associated deaths worldwide (Khan and Fatima 2019), nowadays, colon carcinoma is still difficult to cure, so its morbidity and mortality rates remain high (Yu et al. 2019). Consequently, finding the key molecules implicated in colon cancer relapse and metastasis becomes a problem to be urgently solved. As a monomer purified from traditional Chinese medicine herb, curcumin is capable of influencing colon adenocarcinoma from the aspects of cell proliferation inhibition as well as apoptosis facilitation (Sritharan and Sivalingam 2021). NBR2 can inhibit tumour cell invasion besides proliferation (Xue 2013). The Notch1 signalling pathway, as an oncogenic signalling pathway, can regulate the differentiation of colonic epithelial cells, and its dysregulation induces tumours (Srinivasan et al. 2016). The onset and progression of tumours are jointly mediated by genetic and physiological changes, but the mechanism at the molecular level remains to be elaborated. Hence, the molecular mechanism by which curcumin affected the proliferation of colon cancer cell line SW480 was explored in this study from the perspective of targeted binding of NBR2 to Notch1.

MiRNAs and lncRNAs play crucial roles in regulating the biological functions of cells, the former of which mainly regulates the expressions of target proteins, and the latter affects functional proteins in many ways (Liu et al. 2020). LncRNAs play different roles in tumorigenesis, and its dysregulation is closely related to tumorigenesis (Hang et al. 2015). Highly expressed in colorectal cancer tissues, Notch1 is a potential biomarker for prognostic prediction and treatment (Zhang and Wang 2020). At present, the correlation between NBR2 and Notch1 in colon cancer and their influence on tumour metastasis have not been reported yet. Yang (2015) found that curcumin inhibited colorectal cancer cell proliferation but promoted its apoptosis by means of down-regulating the expressions of Notch1-related proteins. Herein, curcumin at different concentrations dose-dependently inhibited cell proliferation together with Notch1 expression. However, NBR2 expression in SW480 cells climbed up in curcumin treatment groups concentration-dependently. It was predicted through the RNAInter website that NBR2 interacted with Notch1, and the physical binding between NBR2 and Notch1 was confirmed by co-immunoprecipitation assay, conforming to the findings published previously (Cai et al. 2019).

The mechanism related to colon cancer metastasis is highly complicated, involving EMT of cancer cells, invasion and adhesion ability, and angiogenesis. Particularly, EMT is not only the key initiation step of tumour metastasis but also a dominant player in colon cancer progression. Gao et al. (2019) probed into lncRNA NBR2 for its impacts on non-small cell lung cancer as well as its possible molecular mechanism, and found that NBR2 inhibited the progression of EMT by regulating the Notch1 pathway in this disease. Besides, as reported by Zhou et al. (2017), genistein reversed EMT by inhibiting the Notch1/NF-êB/E-cadherin pathway to inhibit the migration of colon cancer cells, which was realised through up-regulating E-cadherin and down-regulating N-cadherin. In this study, after curcumin treatment, the protein expression level of Vimentin was down-regulated whereas that of E-cadherin was up-regulated by curcumin depending on its concentration ($P<0.05$). After transfection, the NBR2 group displayed a lower Notch1 expression than the NC group, but the NBR2+Notch1 group presented a higher Notch1 expression than the NBR2 group. Moreover, overexpressed Notch1 reversed the suppression of Notch1 by NBR2 overexpression, enhancing SW480 cell propagation. Furthermore, overexpressing Notch1 alleviated the down-regulation of NBR2 overexpression on Notch1 in curcumin treatment groups, that is, the function of NBR2 in repressing cell proliferation was deteriorated by Notch1 overexpression.

CONCLUSIONS

In conclusion, curcumin can suppress the proliferation of colon cancer SW480 cells through up-regulating NBR2. Overexpressing Notch1 reverses the inhibitory effects of curcumin on colon cancer cell proliferation, probably through EMT.

RECOMMENDATIONS

This study was conducted using colon cancer SW480 cells. To further validate the role of curcumin, different colon cancer cell lines, animal models and clinical samples should be tested.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BCA: Bicinchoninic acid
DMEM: Dulbecco's modified Eagle medium
EMT: epithelial-mesenchymal transition
lncRNA: long non-coding ribonucleic acids
MTT: methyl thiazolyl tetrazolium
qRT-PCR: quantitative real-time polymerase chain reaction

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