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Curcumin suppresses the proliferation of gastric cancer cells by downregulating H19

GAO LIU[1*], TIAN XIANG[2*], QUAN-FENG WU[1] and WEI-XING WANG[3]

[1]Department of Gastrointestinal Surgery; [2]Department of Clinical Laboratory Center, Central Hospital of Enshi Autonomous Prefecture, Enshi Clinical College of Wuhan University, Enshi, Hubei 445000; [3]Department of Hepatobiliary and Laparoscopic Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. Curcumin, a major phytochemical in turmeric, inhibits the proliferation of many types of solid cancer cells by enhancing p53 expression. However, the long non-coding RNA H19 directly inhibits p53 activation and thus promotes gastric cancer progression. The aim of this study was to assess the role of H19 in curcumin-induced proliferative inhibition of gastric cancer. The gastric cancer cell line SGC-7901 was treated with curcumin at different concentrations and time points. The effect of curcumin on proliferation was assessed using cell counting kit-8 assays and flow cytometry with Ki67 staining. In addition, H19 expression was quantified by reverse transcription-quantitative polymerase chain reaction, and apoptosis was evaluated by flow cytometric detection of Annexin V and propidium iodide double staining. The protein expression of p53, B-cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax) and c-Myc in curcumin-treated cells was detected by western blotting. The present study demonstrated that curcumin inhibited the proliferation of SGC7901 cells and suppressed H19 expression in a concentration-dependent manner, while p53 expression was enhanced. Ectopic expression of H19 in SGC7901 cells reversed curcumin-induced proliferative inhibition and downregulated p53 expression. Furthermore, while curcumin induced cell apoptosis and enhanced the expression ratio of Bax/Bcl-2, which are downstream molecules of p53, ectopic expression of H19 inhibited curcumin-induced cell apoptosis. In addition, curcumin decreased the expression of the c-Myc oncogene, and exogenous c-Myc protein reversed the curcumin-induced downregulation of H19 expression. These results suggested that curcumin inhibits the proliferation of gastric cancer cells by downregulating the c-Myc/H19 pathway. Therefore, curcumin may be considered a novel therapeutic strategy to inhibit gastric cancer cell growth.

Correspondence to: Professor Wei-Xing Wang, Department of Hepatobiliary and Laparoscopic Surgery, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: wang_weixing1956@163.com

[*]Contributed equally

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Introduction

Curcumin, which is commonly called diferuloylmethane, is derived from *Curcuma longa*, a plant of the ginger family (1). Extensive research over the last half century has revealed the therapeutic potential of curcumin in tumor progression, including inducing apoptosis, inhibiting angiogenesis and enhancing susceptibility to chemotherapy and radiotherapy (1,2). Furthermore, the anticancer effect of curcumin has been confirmed in a number of clinical trials, in which is has been used as a natural chemoprevention agent in colorectal and pancreatic cancer (3-5). Accumulating evidence suggests that curcumin has a diverse range of molecular targets, including c-Myc, cyclooxygenase-2, Notch1, nuclear factor-κB and p53 (2,6-8).

The tumor suppressor p53 plays a pivotal role in the etiology of human cancers; it not only controls the cellular proliferation of tumor cells, but is also capable of inducing cell apoptosis (9). Previous studies reported that curcumin induced p53 expression in prostate cancer, B-cell lymphoma (Bcl) cells and breast cancer, and thereby activated the pro-apoptotic downstream genes p21 and Bcl-2-associated X protein (Bax) and inhibited Bcl-2 (anti-apoptosis) expression to induce apoptotic progress (2,10,11). Furthermore, curcumin induced cell-cycle arrest by downregulating cyclin D1 expression (2,10,11).

In gastric cancer, curcumin attenuated *in vivo* tumor growth induced by N-methyl-N-nitrosourea by downregulating the expression of cyclin D1 in tumor cells (12). In *in vitro* studies, curcumin induced cell apoptosis by reducing Bcl-2 expression or enhancing reactive oxygen species production, and induced a G1 cell cycle arrest by downregulating cyclin D1 expression (12-15). Activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway was also inhibited by curcumin, and played a role in promoting cell apoptosis (16). Although Bcl-2 and cyclin D1 are downstream molecules of p53 (17), and LY294002 (a PI3K inhibitor) was shown to induce p53 expression and p53-dependent apoptosis in gastric cancer cells by

inhibiting the activation of PI3K/AKT signaling (18), there is not yet sufficient evidence to confirm that curcumin regulates p53 expression in gastric cancer cells.

The long non-coding RNA (lncRNA) H19 is produced from the paternally imprinted H19 gene and is considered an oncogenic lncRNA in various cancers (19-22). Furthermore, previous studies have reported that H19 is abnormally upregulated in gastric cancer (23-25) and contributes to cellular proliferation by directly inactivating p53 (26). Notably, curcumin downregulated H19 gene transcription and c-Myc expression in human tumor cells (2,27,28). In addition, the c-Myc oncogene was shown to directly induce H19 expression by binding to the H19 promoter, and thereby promoted the proliferation of gastric cancer cells (29,30).

The present study aimed to determine whether curcumin suppresses the proliferation of gastric cancer cells by regulating c-Myc/H19/p53 signaling. It was confirmed that curcumin inhibited the proliferation of gastric cancer cells, suppressed H19 and c-Myc expression, and enhanced p53 expression in a time- and concentration-dependent manner. Overexpression of H19 in gastric cancer cells reversed curcumin-induced cell apoptosis and the inhibitory effect on cell proliferation, as well as decreasing p53 expression in the presence of curcumin. Furthermore, exogenous c-Myc enhanced H19 expression in gastric cancer cells in the presence of curcumin. Together, these results suggested that curcumin exploited a novel mechanism to inhibit gastric cancer cell growth.

Materials and methods

Reagent and cell culture. Curcumin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck Millipore) and stored at -20˚C until use. Active human c-Myc full-length protein was purchased from Abcam (Cambridge, MA, USA) and added to media for a final concentration of 5 μg/ml (31). The human gastric cancer cell line SGC7901 and the immortalized human gastric epithelial mucosa cell line GES-1 were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured in a humidified incubator containing 5% CO_2 at 37˚C. For the c-Myc protocol, recombinant human c-Myc protein (5 μg/ml) was added to the media of SGC7901 cells in the presence of 50 μM curcumin.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNase-free DNase I (Thermo Fisher Scientific, Inc.) treatment was performed to remove any contaminating DNA. RT-qPCR was performed using the ReverTra Ace-α first-strand cDNA synthesis kit and the SYBR Green Real-time PCR Master mix kit (both Toyobo Co., Ltd., Osaka, Japan). For mRNA detection, the primers used in this study were as follows: H19 forward, 5'-TACAACCACTGCACTACCTG-3' and reverse, 5'-TGGAATGCTTGAAGGCTGCT-3' (32); and GAPDH (as an internal control) forward, 5'-ACCTGA CCTGCCGTCTAGAA-3' and reverse, 5'-TCCACCACC CTGTTGCTGTA-3' (33). The ABI StepOne Plus (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform qPCR. PCR reactions were performed at 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Each experiment was performed in triplicate. The relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (34).

Transfection. H19 cDNA (GenBank accession no. NR_002196.1) was inserted into the multiple cloning sites of the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.), as described previously (33). A total of $1x10^5$ cells were plated onto 24-well plates for 24 h and then transfected with 0.5 μg plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. The cells were then subjected to RNA/protein extraction or further functional assays.

Cell proliferation assay. Cell proliferation assays were performed using a Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China), as described previously (35). Briefly, SGC7901 cells ($1x10^4$ cells/well) were plated onto 96-well plates, and then treated with curcumin or pre-transfected with pcDNA3.1-H19 or empty vector for 48 h. The number of cells per well was detected by measuring the absorbance (450 nm) of reduced WST-8 at various time points using the SpectraMax® i3x microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell apoptosis. Evaluation of cell apoptosis was performed using the FITC Annexin V Apoptosis Detection kit with PI (BioLegend, Inc., San Diego, CA, USA). Briefly, the cells were washed twice with cold BioLegend's Cell Staining Buffer, and then resuspended in Annexin V Binding Buffer at a concentration of $0.25\text{-}1.0x10^7$ cells/ml. This suspension (100 μl) was stained with 5 μl FITC/Annexin V and 10 μl PI, after which the cells were gently vortexed and incubated for 15 min at room temperature (25˚C) in the dark. Subsequently. 400 μl Annexin V Binding Buffer was added to each tube, which were analyzed by flow cytometry.

Ki67 staining. The cells were washed twice with PBS by centrifugation at 350 x *g* for 5 min at 4˚C, and then resuspended in 3 ml cold 70% ethanol and incubated at -20˚C for 1 h. Subsequently, the cells were resuspended in 100 μl PBS in the presence of phycoerythrin-conjugated anti-human Ki67 antibody (1:20; cat. no., 350504; BioLegend, Inc.), and then incubated at room temperature in the dark for 30 min. Next, 500 μl PBS was added to resuspend the cells for flow cytometric analysis.

Western blotting. Proteins were extracted from the cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and were quantified using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membrane was

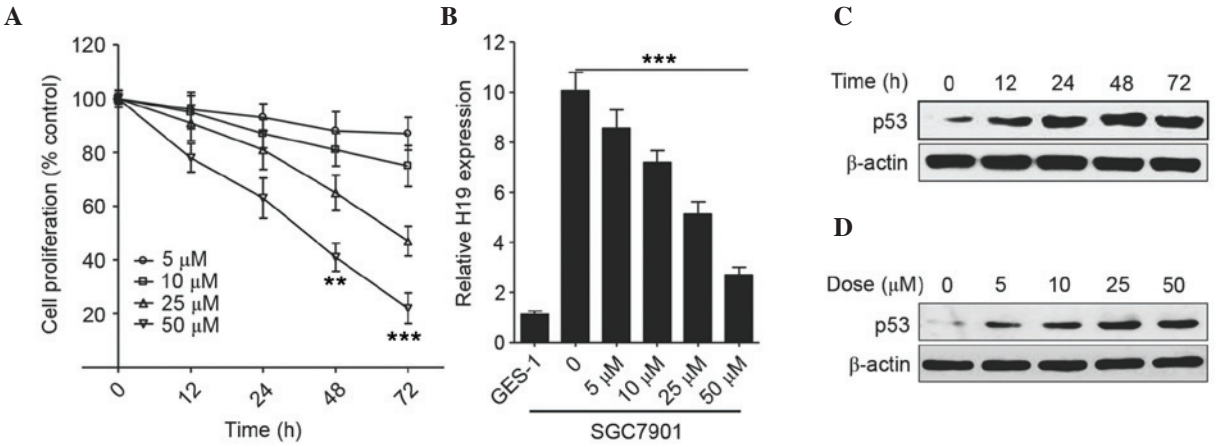

Figure 1. Effect of curcumin on cell proliferation and H19 and p53 expression. SGC7901 cells were treated with different concentrations of curcumin and then collected at different time points. (A) Cell proliferation was assessed using cell counting kit-8 assays. Data are shown as the mean ± SD of three separate experiments performed in duplicate. (B) The relative mRNA expression levels of H19 were determined by reverse transcription-quantitative polymerase chain reaction. H19 levels were normalized to the GAPDH level in GES-1 cells. Data are shown as the mean ± SD of three separate experiments performed in duplicate. (C) Western blotting was performed to detect p53 expression in the presence of curcumin. (D) Western blotting was performed to detect p53 expression in the presence of various concentrations of curcumin. **P<0.01; ***P<0.0001. SD, standard deviation.

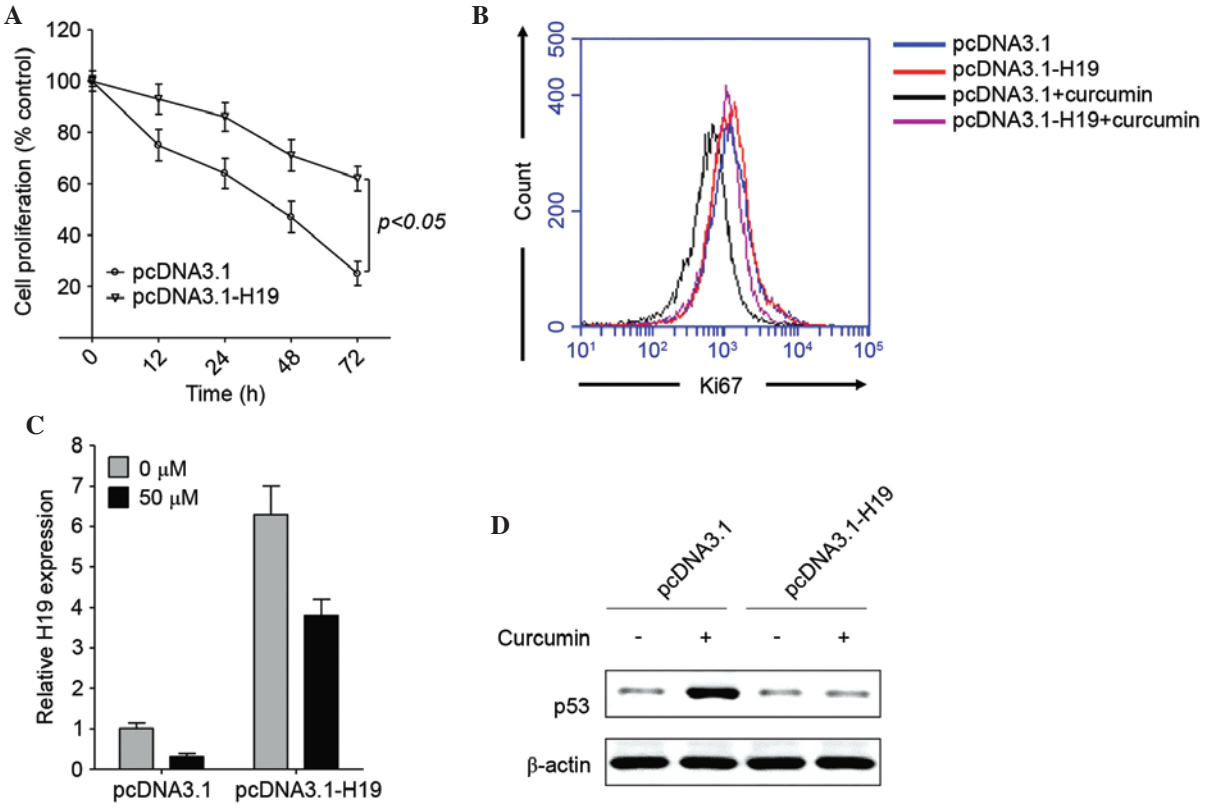

Figure 2. Ectopic expression of H19 enhances cell proliferation in the presence of curcumin. (A) SGC7901 cells were plated into 96-well plates and transfected with pcDNA3.1-H19 plasmid or empty vector control, after which the cells were treated with 50 μM curcumin for various durations. Cell proliferation was determined using cell counting kit-8 assays. SGC7901 cells were plated into 24-well plates and transfected with pcDNA3.1-H19 plasmid or empty vector control, and then cells were treated with or without 50 μM curcumin for 72 h. (B) Ki67 expression was detected by flow cytometry. The relative expression levels of (C) H19 mRNA and (D) p53 protein were determined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Data are shown as the mean ± standard deviation of three separate experiments performed in duplicate.

blocked with 5% nonfat milk and incubated with diluted antibodies at 4°C overnight. Primary antibodies against p53 (1:1,000; cat. no. 1C12), Bax (1:1,000; cat. no. D2E11), Bcl-2 (1:1,000; cat. no. 50E3), c-Myc (1:1,000; cat. no. D84C12) and β-actin (1:1,000; cat. no. 13E5) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no., sc-2055; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 1 h. The immunoreactive bands were visualized using

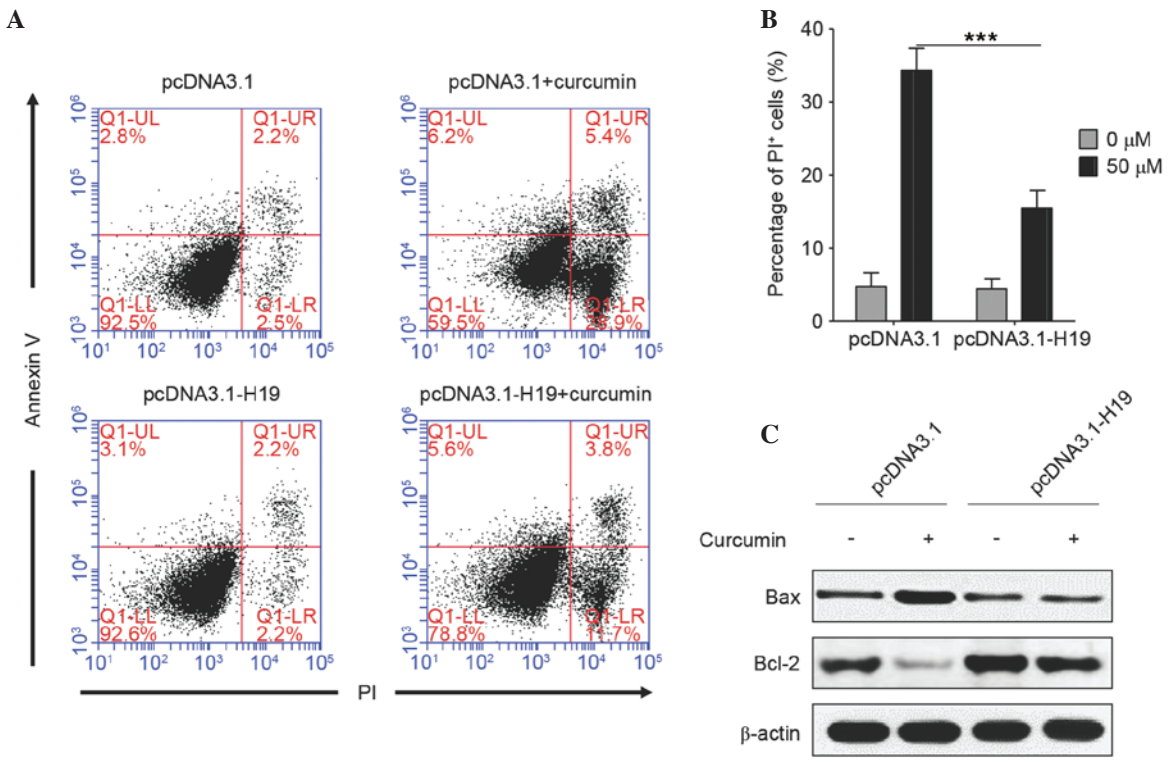

Figure 3. Ectopic expression of H19 inhibits cell apoptosis induced by curcumin. SGC7901 cells were plated onto 24-well plates and transfected with pcDNA3.1-H19 plasmid or empty vector control, after which the cells were treated with or without 50 μM curcumin for 48 h. (A) Cell apoptosis was determined by Annexin V and PI double staining. (B) PI positive cells were calculated. Data are shown as the mean ± standard deviation of three separate experiments performed in duplicate. (C) Bax and Bcl-2 expression was determined by western blotting. ***P<0.0001. PI,propidium iodide; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

the Immobilon™ Western Chemiluminescent HRP Substrate (EMD Millipore) and the UVP Bioimaging system (UVP, Inc., Upland, CA, USA).

Statistical analysis. All experiments were performed three times. Data are presented as the mean ± standard deviation and analyzed using GraphPad Prism 5.00 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences among the groups were assessed by one-way analysis of variance followed by Neuman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin inhibits gastric cancer cell proliferation and H19 expression. Initially, the effect of curcumin on the proliferation of the gastric cancer cell line SGC7901 was analyzed by CCK-8 assays in the presence of various concentrations of curcumin for 12, 24, 48 and 72 h. As shown in Fig. 1A, curcumin inhibited the growth of SGC7901 cells in a concentration- and a time-dependent manner. In comparison with the untreated cells, cell proliferation was significantly inhibited after 48 h of treatment with 50 μM curcumin (P<0.01). The relative mRNA expression level of H19 was decreased in a dose-dependent manner following treatment with various concentrations of curcumin (Fig. 1B), and, as compared with SGC7901 cells in the absence of curcumin, showed the lowest level at 50 μM (P<0.0001). As for p53 expression in SGC7901 cells, curcumin markedly increased the expression level of p53 after 12 h (Fig. 1C and attained a peak at 48 h following treatment with 25 μM curcumin (Fig. 1D).

Ectopic expression of H19 reverses curcumin-mediated inhibition of proliferation. To further elucidate the role of H19 in curcumin-induced proliferative inhibition of gastric cancer cells, H19 was overexpressed in SGC7901 cells, which were subsequently treated with 50 μM curcumin, as this concentration of curcumin induced the highest level of proliferative inhibition (Fig. 1A). As compared with the empty vector control, ectopic expression of H19 significantly enhanced cell proliferation in the presence of curcumin, as determined using the CCK-8 assay (P<0.05; Fig. 2A) or Ki67 staining (Fig. 2B), which is a nuclear antigen only present in proliferating cells (36). In pcDNA3.1-H19-transfected cells, curcumin downregulated H19 expression (Fig. 2C), but did not enhance p53 expression (Fig. 2D). As H19 directly binds to p53 and deactivates p53 expression (26), curcumin may depend on the inhibition of H19 expression to enhance the tumor-suppressive activity of p53. These results suggest that curcumin enhances p53 expression by downregulating H19 expression.

Ectopic expression of H19 reverses curcumin-induced cell apoptosis. Subsequently, the role of H19 in curcumin-induced apoptosis of SGC7901 cells was analyzed. As shown in Fig. 3A and B, there was no significant difference in cell apoptosis between the cells transfected with empty vector and pcDNA3.1-H19 (PI-positive, 4.7 vs. 4.4%), which suggested that plasmid transfection did not induce a difference in cell

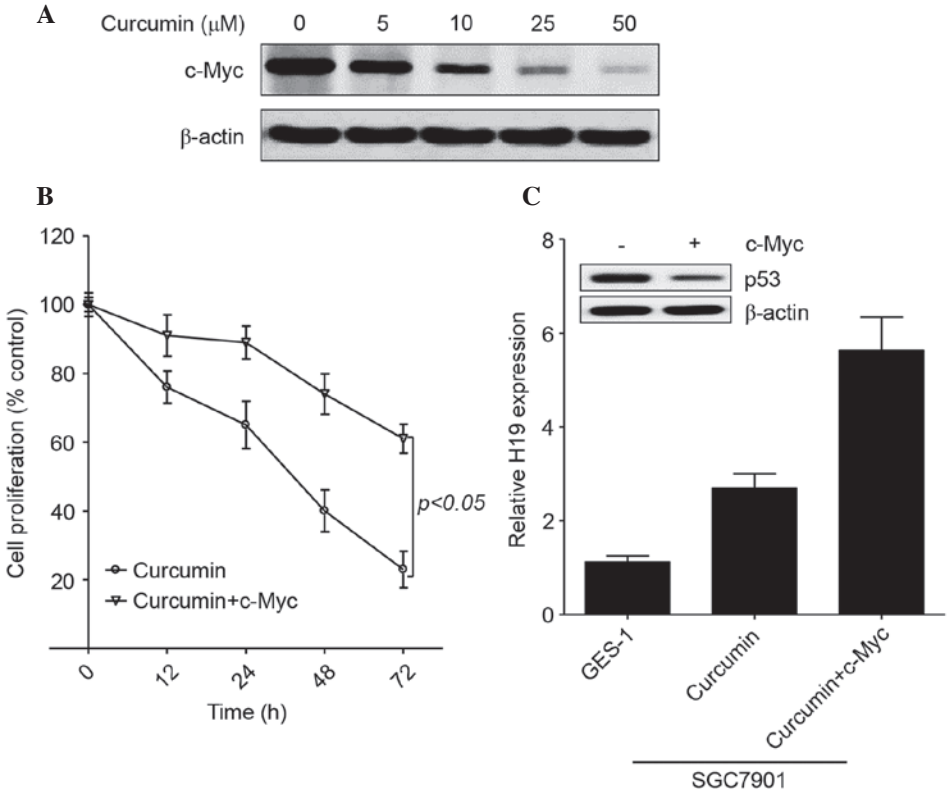

Figure 4. Effect of c-Myc on H19 and p53 expression in curcumin-treated cells. (A) SGC7901 cells were plated into 24-well plate and treated with different concentrations of curcumin for 48 h, after which the protein expression of c-Myc was determined by western blotting. (B) SGC7901 cells were plated into 96-well plates and treated by 50 μM curcumin with or without exogenous c-Myc (5 μgml) for various durations. Cell proliferation was determined by cell counting kit-8 assays. Data are shown as the mean ± SD of three separate experiments performed in duplicate. (C) SGC7901 cells were plated into 24-well plates and treated with 50 μM curcumin with or without exogenous c-Myc (5 μgml) for 48 h. The relative mRNA expression levels of H19 were determined by reverse transcription-quantitative polymerase chain reaction. H19 levels were normalized to the GAPDH level in GES-1 cells. Data are shown as the mean ± SD of three separate experiments performed in duplicate. Protein expression of p53 was determined by western blotting.

apoptosis. Curcumin significantly induced the apoptosis of cells transfected with empty vector (~34.3% were PI-positive), whereas, in H19-transfected cells, the percentage of apoptotic cells was ~15.5%, which was significantly lower compared with cells transfected with empty vector (P<0.0001). An increase in the ratio of Bax/Bcl-2 is known to initiate apoptosis (17); it was noted that curcumin markedly increased Bax expression and decreased Bcl-2 expression in empty vector-transfected cells, while this effect was almost diminished in H19-overexpressed cells (Fig. 3C). These results suggest that curcumin induces cell apoptosis by downregulating H19 expression.

c-Myc enhances H19 expression in curcumin-treated gastric cancer cells. As an oncogene, the expression of c-Myc has been shown to be upregulated in patients with gastric cancer and to induce H19 expression in gastric cancer cells (29,30). Furthermore, curcumin inhibited c-Myc expression in Bcl and skin cancer (2,28). Therefore, the present study further evaluated the role of c-Myc in regulating H19 expression in the presence of curcumin. As shown in Fig. 4A, curcumin markedly decreased c-Myc expression in gastric cancer cells in a concentration-dependent manner. Similar to H19, exogenous c-Myc induced cell proliferation in the presence of 50 μM curcumin (Fig. 4B). In addition, exogenous c-Myc enhanced H19 expression and decreased p53 expression in curcumin-treated SGC7901 cells (Fig. 4C). These results confirm that curcumin inhibits H19 expression by regulating c-Myc expression in gastric cancer.

Discussion

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer-associated mortality worldwide, with an estimated 952,000 new cases diagnosed and 723,000 deaths registered in 2012 (37). Previous studies have demonstrated that H19 plays an oncogenic role in gastric cancer and predicts a poor prognosis in patients with gastric cancer (25,26,29,33,38). However, an agent that is able to downregulate H19 expression in tumor cells has rarely been reported (39). The present study demonstrated that curcumin, a naturally occurring phytochemical, was able to inhibit H19 expression in gastric cancer cells and thereby induce apoptosis and inhibit cellular proliferation.

Curcumin is able to suppress the proliferation and survival of cancer cells by directly or indirectly binding to various targets, including transcription factors, growth factors and several proteins that are involved in cell signal transduction pathways (40). c-Myc is an important oncogene that has been shown to be downregulated by curcumin (2). Similarly, the present study observed that curcumin decreased c-Myc expression in a concentration-dependent manner in gastric cancer. c-Myc regulates numerous gene targets

that subsequently execute its many biological activities, including cell proliferation, transformation, angiogenesis and apoptosis (41). Furthermore, elevated expression of c-Myc correlates with a poor prognosis in various cancers, including head and neck squamous cell carcinoma, breast cancer and hepatocellular carcinomas (42-45). The present study also demonstrated that exogenous c-Myc was able to reverse curcumin-induced proliferative inhibition in gastric cancer cells.

Previous studies have indicated that c-Myc promotes cancer progression by upregulating tumor-promotive lncRNAs, including prostate cancer gene expression marker 1 and HOX transcript antisense RNA (46,47). In addition, c-Myc has been reported to directly bind to the promoter of H19 in order to induce its expression and potentiate tumor progression in primary breast and lung carcinomas (30). In gastric cancer, c-Myc has been shown to induce H19 expression, and its expression was positively correlated with H19 expression in gastric cancer patients (29). The present study demonstrated that exogenous c-Myc enhanced H19 expression in the presence of curcumin, which provided evidence to explain how curcumin inhibited H19 expression, and provides a direct molecular link between curcumin and H19. However, whether c-Myc is indispensable for curcumin to regulate H19-mediated p53 deactivation still needs to be clarified in future.

The role of H19 in the progression of gastric cancer may be due to its association with p53 (26). p53, which is an important tumor suppressor, plays a pivotal role in inhibiting the proliferation and inducing the apoptosis of cancer cells (11). In the present study, curcumin significantly enhanced p53 expression, and simultaneously induced cell apoptosis and inhibited proliferation of gastric cancer cells. Conversely, ectopic expression of H19 abrogated curcumin-induced p53 expression, and the following effects on proliferation and apoptosis of cancer cells.

In conclusion, the major findings of this study can be summarized as follows: i) Curcumin inhibits H19 expression in gastric cancer cells; ii) H19 plays a pivotal role in curcumin-induced proliferative inhibition and apoptosis of gastric cancer cells; and iii) c-Myc can be downregulated by curcumin and is an important mediator between curcumin and H19. To the best of our knowledge, the present study demonstrated, for the first time, a novel mechanism by which curcumin exploits a lncRNA to inhibit gastric cancer growth. Therefore, curcumin may be considered a value therapeutic strategy for the treatment of gastric cancer.

Acknowledgements

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