microRNA-16 represses colorectal cancer cell growth *in vitro* by regulating the p53/survivin signaling pathway

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Received November 1, 2012; Accepted December 21, 2012

DOI: 10.3892/or.2013.2262

Abstract. Dysregulated expression of microRNAs (miRNA) is a hallmark of cancer. miR-16 has been reported to be downregulated and to act as a tumor suppressor in different cancer types. In the present study, we sought to investigate the possible roles and mechanisms of miR-16 and its relationship with p53 and survivin in CRC cells. We showed that miR-16 was downregulated in 67% of CRC tissues and was correlated with the degree of histological differentiation. Experiments *in vitro* showed that overexpression of miR-16 inhibited the proliferation and induced apoptosis of CRC cells through the intrinsic apoptosis pathway. We further showed that miR-16 repressed survivin expression at both the mRNA and protein levels and the *survivin* gene was a direct target of miR-16. In addition, miR-16 reduced p53 expression and p53 increased miR-16 levels, with downregulation of miR-16 targets survivin, cyclin D1 and CDK6. Our findings suggest that miR-16 represses colorectal cancer cell growth *in vitro* by regulating the p53/survivin signaling pathway. Our findings provide further evidence for the involvement of dysregulated miRNAs in CRC, and miR-16 could serve as a molecular target for CRC therapy.

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Abbreviations: CRC, colorectal cancer; miR, microRNA; CLL, chronic lymphocytic leukemia; CCK-8, Cell Counting Kit-8; IAP, the inhibitor of apoptosis

Key words: microRNA, miR-16, cell proliferation, apoptosis, colorectal cancer, survivin, p53

Introduction

microRNAs (miRNAs) are a group of small non-coding RNAs 21-25 nucleotides in length that negatively regulate the expression of targeted genes in animals, plants and viruses by interacting with complementary sites in the 3' untranslated region (3'UTR) and degrading mRNA or suppressing the translation of target genes (1). More than 60% of human protein-encoding genes are under selective pressure to maintain pairing with miRNAs (2), suggesting that miRNAs may play important roles in a wide array of physiological and pathological processes, including human oncogenesis, cell differentiation, proliferation and apoptosis. The dysregulation of these processes is also a hallmark of cancer. Mutations affecting miRNAs or their functional interactions with oncogenes and tumor-suppressor genes may be involved in tumorigenesis.

miR-16-1 is located at chromosome 13q14, which is deleted in 68% of chronic lymphocytic leukemia (CLL) cases (3), and is downregulated in most solid tumors (4-7). miR-16 inhibits cell proliferation and induces cell cycle arrest, and increases cell apoptosis by negatively regulating Bcl2 in CLL, prostate and hepatocellular carcinoma cancer cells (6,8,9). Moreover, several studies have shown that p53, as a nuclear protein that is essential for cell cycle control, DNA repair and induction of apoptosis under many stresses, also enhances the maturation of several miRNAs at the post-transcriptional levels, including miR-16 (10,11). The p53 protein has also been shown to play important roles in the oncogenesis of colon cancer. These findings indicate that miR-16, a miRNA related to p53, has a critical function in human carcinogenesis. However, the possible roles and mechanisms of miR-16 and its relationship with p53 in CRC cells are still not well established.

In this study, we sought to investigate the expression of miR-16 in CRC and normal adjacent tissues and elucidate the effect of miR-16 on the growth of CRC cells *in vitro* and examine its downstream target. We found that miR-16 was downregulated in 67% of CRC tissues, and overexpression of miR-16 inhibited cell proliferation and induced cell apoptosis in CRC cells. We further identified the oncogene survivin as a direct and functional target of miR-16. Furthermore, we found that miR-16 regulated the p53/survivin signaling pathway in CRC cells *in vitro*. Our findings provide further evidence for

the implication of dysregulated miRNAs in CRC, and miR-16 may serve as a molecular target for CRC, which to date has a dismal outcome with limited therapeutic options.

Materials and methods

Cells and acquisition of tissue specimens. Human CRC cell lines HCT116 and LoVo were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Surgically excised tumor specimens and normal adjacent tissues from 52 patients with World Health Organization (WHO) grade I-IV CRC were collected at the Department of Gastroenterology, Huizhou Center Hospital, Huizhou, China. Each tumor specimen was divided into two parts. One part was immediately snap-frozen in liquid nitrogen and stored at -80˚C, the other part was used for pathological assesment by which the tumor differentiation was graded as well, moderate or poor (including undifferentiated) (12). The protocol for this study and acquisition of tissue specimens were approved by the local institutional review boards at the affiliated institutions of the authors. Human tissue acquisition and use in this study complied with the National Regulations on the Use of Clinical Samples in China.

Quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using TaqMan MicroRNA Reverse Transcription kit and miR-16 RT primers (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out with TaqMan Universal Master Mix II and miR-16 TaqMan MicroRNA Assay Mix on an ABI PRISM 7500 (Applied Biosystems). miR-16 expression was normalized against U6. In addition, cDNA was synthesized from 1.5 μg total RNA with M-MLV reverse transcriptase (Invitrogen) in a 20-μl volume, and mRNA levels of survivin were detected using a SYBR-Green I Mix (Roche, Switzerland) in a 20-μl reaction volume on Light Cycler 480II/96 (Roche) as instructed by the manufacturer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Data analysis was carried out using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for real-time PCR were: survivin, 5'-CCACCGCATCTCTACATTCA-3' (sense) and 5'-TATGTTCCTCTATGGGGTCG-3' (antisense); GAPDH, 5'-GTCAACGGATTTGGTCGTATTG-3' (sense) and 5'-CTCCTGGAAGATGGTGATGGG-3' (antisense).

miRNA mimics, inhibitors, small interfering RNA (siRNA), plasmids and transfections. miR-16 mimics and inhibitors and scrambled controls were purchased from GenePharma (Shanghai, China). The sequences of these miRNAs were: miR-16 mimics, 5'-UAGCAGCACGUAAAUAUUGGCG-3'; miR-16 inhibitors, 5'-CGCCAAUAUUUACGUGCUGCUA-3'; scrambled control, 5'-UUCUCCGAACGUGUCACGUTT-3'. The p53 gene was amplified from pGEMT-p53. The amplified p53 gene products were digested by *Not*I and *Bam*HI and inserted into pcDNA3.1 to obtain pcDNA3.1-p53. Cells were transfected with appropriate miRNA, siRNA oligonucleotides and plasmids using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. The medium was replenished 6 h after transfection.

CCK-8 cell proliferation assays. Cell proliferation was detected by Cell Counting Kit-8 (CCK-8) (Beyotime, China) according to the manufacturer's protocol. Briefly, cells were plated in 96-well plates at $0.5x10^4$ cells/well. CCK-8 reagent (10 μl) was added at 0, 24, 48 and 72 h after transfection, and after a 1-h incubation at 37˚C, the optical density (OD) was read at 450 nm by a microplate luminometer reader.

Apoptosis assays. Apoptosis assays were performed using a commercial Annexin V apoptosis detection kit according to the manufacturer's protocol (BD Biosciences). Briefly, transfected cells were collected, washed with PBS and resuspended in binding buffer containing 10 mM HEPES (pH 7.4); 2.5 mM $CaCl_2$, and 140 mM NaCl. Annexin V-PE and 7-AAD were added and FACS was performed after 15 min of incubation at room temperature. Experiments were performed in triplicates and at least three times independently.

Luciferase assays. The 3'-UTRs of the target gene *survivin* were amplified by PCR using the following primers: 5'-atctGGTACCGGAGAAAGTGCGCCGTGCCA-3' and 5'-tcctAAGCTTGRGGAAGGCTCTGCCCACGC-3'. The PCR products were then gel purified, digested and inserted into pGL3-basic vector (Promega, Madison, WI, USA). Target site mutations were generated using the PCR products with the appropriate primers containing point substitutions. The sequences were confirmed by sequencing.

We performed the luciferase assays using HCT116 cells transiently transfected with 0.1 μg reporter plasmid and 0.65 pmol miRNA mimics or control miRNA in 96-well plates or with *Renilla* constructs (as an internal control). Luciferase assays were performed 48 h later using the Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega). All luciferase activity readings were normalized relative to the activity of the *Renilla* luciferase control. All experiments were performed in triplicate.

Western blot assays. Total protein was extracted using a commercially available whole cell lysis buffer (Beyotime). Immunoblotting was performed as previously described (13). The following antibodies were used: anti-survivin and anti-caspase-9 antibodies (Cell Signaling Technology, Beverly, MA, USA), anti-p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH antibodies (Zhongshan Goldenbridge Biotechnology, Guangzhou, China), anti-cyclin D1 and anti-CDK6 antibodies (Santa Cruz Biotechnology), anti-capase-3, and anti-caspase-8 antibodies (BD Biosciences). Protein bands were visualized using ECL substrates (Millipore, USA).

Statistical analysis. Data are expressed as means ± SD and analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The Wilcoxon or t-test was used for comparing two groups, and one-way ANOVA was used for comparing multiple groups where the Bonferroni or Tamhane's T2 test was used

to compare differences between groups. A P-value <0.05 was considered to indicate a statistically significant result.

Results

miR-16 is downregulated and is correlated with histological differentiation in primary colorectal cancer tissues. miR-16 has been shown to be downregulated in CLL, lung, prostate and ovarian cancer (4-7), but whether miR-16 expression in CRC is dysregulated remains to be elucidated. In this study, we sought to examine miR-16 expression in an independent primary CRC cohort. We examined the expression of miR-16 in 52 patients with WHO grade I-IV CRC and normal adjacent tissues by real-time RT-PCR. We found that miR-16 was downregulated in 67% (35/52) of the CRC tissues (Fig. 1), and the relative expression level of miRNA transcripts (median 0.59, 25% quartile 0.33, 75% quartile 1.17) was lower than that of adjacent normal tissues (P=0.043). In addition, miR-16 expression was inversely correlated to histological differentiation (P=0.002). miR-16 levels expressed as a median (25-75% quartile) in tumor tissues of poor, moderate and well differentiation were: 0.13 (0.05-0.33), 0.59 (0.36-1.12) and 0.99 (0.52-1.83), respectively.

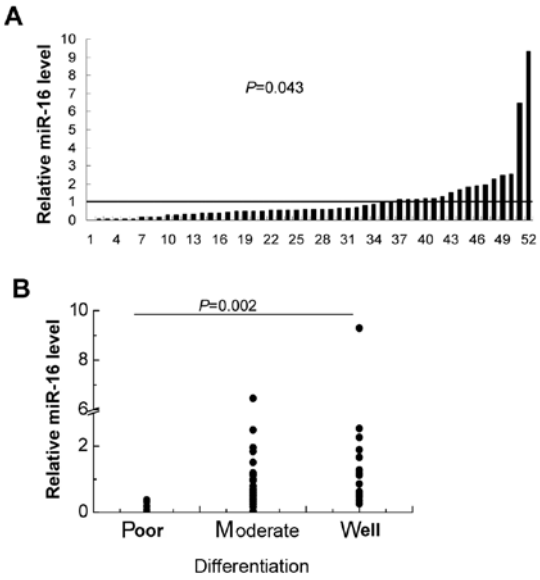

Figure 1. miR-16 is downregulated and correlated to histological differentiation in colorectal cancer (CRC) tissues. (A) miR-16 expression in 52 paired CRC and normal adjacent tissues was examined by real-time RT-PCR and normalized against U6 rRNA. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. A line was drawn to indicate 1-fold difference in expression. The numbers in the X-axis represent individual patients with CRC. (B) miR-16 expression in tumors with different degrees of histological differentiation.

miR-16 overexpression inhibits the proliferation of colorectal cancer cells in vitro. To further elucidate the effect of miR-16 on CRC, we first examined its effect on the proliferation of CRC cells. We transiently transfected HCT116 and LoVo cells with miR-16 inhibitors and mimics. Our RT-PCR assays showed that cells transfected with miR-16 mimics exhibited a markedly higher level of miR-16 than cells transfected with scrambled RNA (259.83±21.63 and 307.46±26.93, respectively) (Fig. 2A and B). On the other hand, miR-16 inhibitors caused a marked reduction in the levels of miR-16 in the transfected cells (0.21±0.03 and 0.32±0.04, respectively) (Fig. 2A and B). We further found that miR-16 inhibitors exerted no apparent effect on the proliferation of HCT116 and LoVo cells while miR-16 mimics caused a significant decrease in the proliferation of the transfected cells at 48- and 72-h post-transfection (Fig. 2C and D).

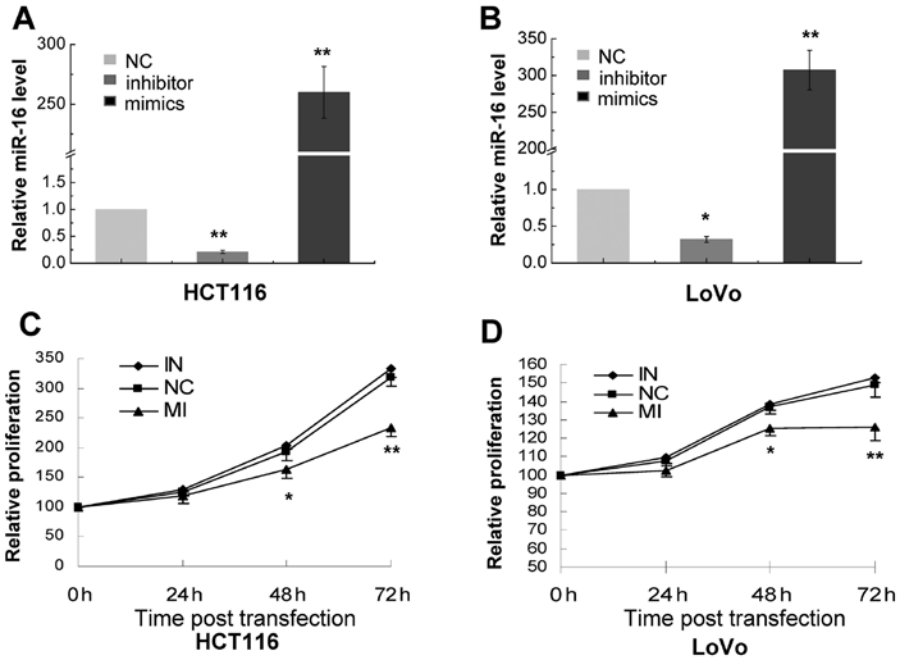

Figure 2. miR-16 inhibits the proliferation of colorectal cancer (CRC) cells. Expression of miR-16 was determined by real-time RT-PCR in (A) HCT116 and (B) LoVo cells 48 h after transfection with miR-16 inhibitors, scrambled control (NC) and miR-16 mimics. Relative cell proliferation was determined by CCK-8 in (C) HCT116 and (D) LoVo cells transfected with miR-16 inhibitors (IN), NC and miR-16 mimics (MI). *P<0.05, **P<0.01. The data shown are representative of 3 independent experiments.

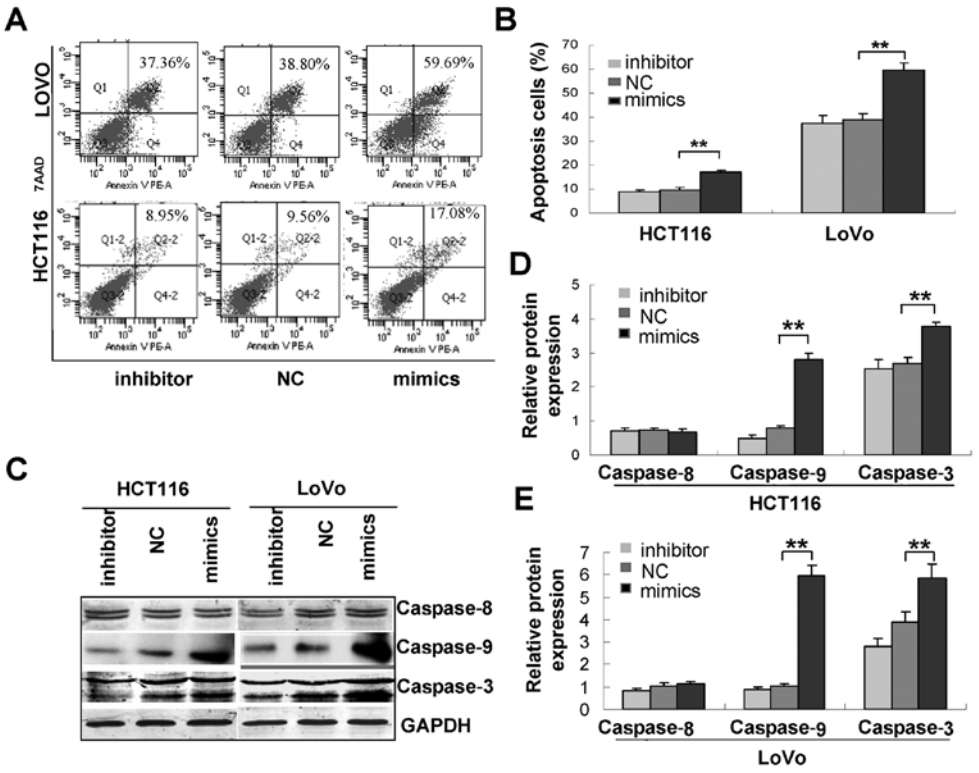

Figure 3. miR-16 induces the apoptosis of colorectal cancer (CRC) cells. (A) Apoptosis was analyzed by flow cytometry in HCT116 and LoVo cells transfected with miR-16 inhibitors, scrambled control (NC) and miR-16 mimics. (B) Graphical representation of the apoptosis of CRC cells by FACS analysis in A. Data are presented as means ± SD of three independent experiments. (C) Cleaved caspase-9, -8 and -3 were detected by western blot assays in HCT116 and LoVo cells transfected with miR-16 inhibitors, NC and miR-16 mimics. (D and E) Graphical representation of the western blot analysis in C. The data shown are representative of 3 independent experiments. **P<0.01.

miR-16 overexpression enhances apoptosis of colorectal cancer cells in vitro. Next, we examined whether miR-16 induces the apoptosis of CRC cells. Flow cytometric analysis of Annexin V-stained cells transfected with miR-16 inhibitors or mimics or scrambled control showed that miR-16 caused a significant increase in the percentage of apoptotic cells from 9.56±1.13 to 17.08±0.64% in HCT116 cells and from 38.80±2.63 to 59.69±2.99% in LoVo cells (P<0.05 in both) while miR-16 inhibitors caused no apparent change in the percentage of apoptotic CRC cells (Fig. 3A and B). Immunoblotting assays further revealed that miR-16 caused a marked increase in the expression of cleaved caspase-9 and -3 (Fig. 3C), suggesting that miR-16 may induce apoptosis through the intrinsic apoptosis pathway.

miR-16 directly targets survivin. Survivin is implicated in regulating the intrinsic apoptotic pathway. We examined whether the *survivin* (also named as BIRC5) mRNA sequence (NM-001012270) contains target sites of miR-16 through RNA22, and the miR-16 binding site was found in the *survivin* sequence (Fig. 4A). We then investigated whether miR-16 affects the protein or mRNA expression of survivin. We found that transfection of CRC cells with miR-16 mimics caused a significant reduction in the survivin mRNA transcript levels (Fig. 4B), a finding that was further confirmed by western blot assays (Fig. 4C), which showed an apparent decrease in the levels of survivin in HCT116 and LoVo cells transfected with miR-16 mimics. Transient transfection of HCT116 cells with the reporter plasmid containing the seed sequence of the 3'-UTR of the *survivin* gene and miR-16 mimics produced a significant reduction in luciferase reporter gene activities (P<0.001 vs. the scrambled control) (Fig. 4D). By contrast, transient transfection of HCT116 cells with the reporter plasmid-containing mutations in the seed sequence of the 3'-UTR of the *survivin* gene (Fig. 4A) and miR-16 mimics did not decrease the activities of the mutant luciferase reporter. These findings suggest that the 3'-UTR of the *survivin* gene was a functional target site of miR-16, which, when overexpressed, suppressed the expression of survivin at both the mRNA and protein levels.

Knockdown of survivin expression by siRNA represses growth and induces apoptosis of colorectal cancer cells in vitro. To further confirm the potential relationship between miR-16 and the downstream target survivin, we knocked down the expression of survivin in CRC cells by transfection with siRNA against survivin (Fig. 5A) and then examined the effect on the proliferation and apoptosis of the transfected cells. Flow cytometry of Annexin V-stained CRC cells indicated that survivin downregulation by siRNA caused a marked increase in the percentage of apoptotic cells (Fig. 5B). CCK-8 assays further showed that survivin downregulation was associated with markedly impaired growth of HCT116 and LoVo cells (Fig. 5C and D), which is consistent with the phenotype of CRC cells overexpressing miR-16.

miR-16 regulates the p53/survivin signaling pathway. It has been reported that p53 is a target of miR-16, and p53 regulates maturation of miR-16 (11,14). We also showed here that survivin

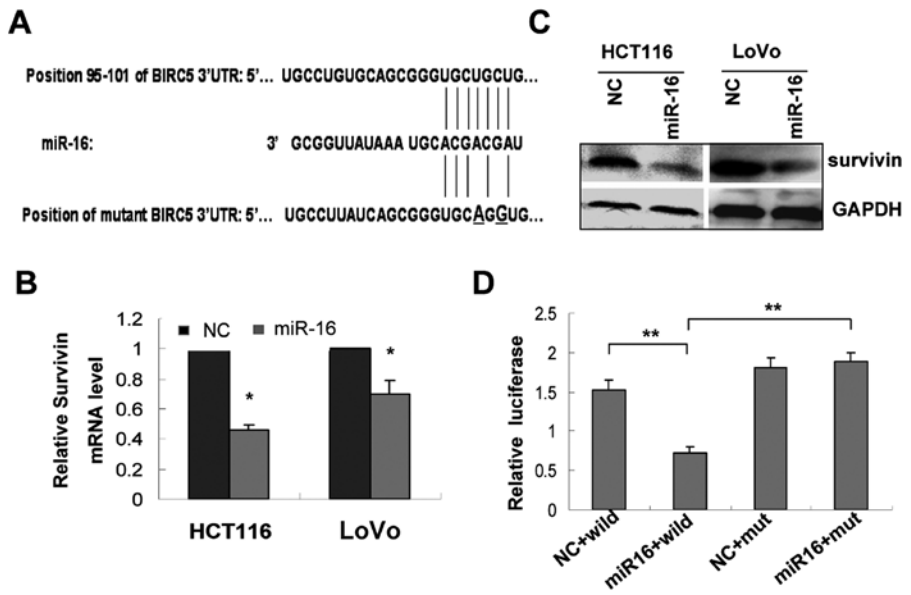

Figure 4. Survivin is a direct target of miR-16. (A) The potential interaction between miR-16 and the putative binding sites in the 3'-UTR of the *survivin* gene (also named as BIRC5) was predicted using RNA22 database. Mutation was generated in the miR-16-binding sequence of the 3'-UTR of the *survivin* gene as indicated (bold and underlined). (B) The expression of survivin mRNA was examined by real-time RT-PCR in HCT116 and LoVo cells transfected with scrambled control (NC) and miR-16 mimics. (C) The expression of survivin was detected by western blot assays in HCT116 and LoVo cells transfected with NC and miR-16 mimics. (D) HCT116 cells were transiently transfected with the reporter plasmid containing the seed sequence or mutated seed sequence of the 3'-UTR of the *survivin* gene and miR-16 mimics. Relative luciferase activities were then determined. Data are expressed as the means ± SD of three independent experiments. *P<0.05, **P<0.01.

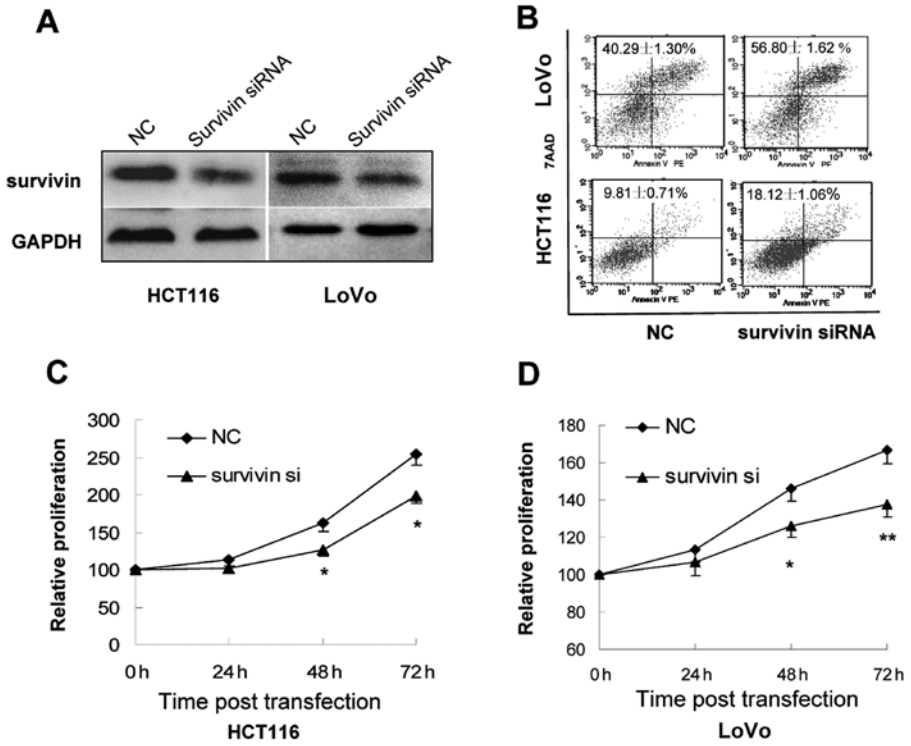

Figure 5. Survivin downregulation by siRNA represses growth and induces apoptosis of colorectal cancer (CRC) cells. (A) Expression of survivin was examined by western blot assays in HCT116 and LoVo cells transfected with scrambled control (NC) and siRNA against survivin. (B) Apoptosis was analyzed by flow cytometry in HCT116 and LoVo cells transfected with NC and siRNA against survivin. Relative cell proliferation was determined by CCK-8 in (C) HCT116 and (D)LoVo cells transfected with NC and siRNA against survivin. *P<0.05, **P<0.01. The data shown are representative of 3 independent experiments.

is a direct target of miR-16. We subsequently performed assays to determine the association of p53, miR-16 and survivin. We first examined p53 expression in cells overexpressing miR-16 and found that miR-16 overexpression noticeably inhibited p53 levels in both HCT116 and LoVo cells, and cells cotransfected with the miR-16 mimics and p53 repressed p53 expression

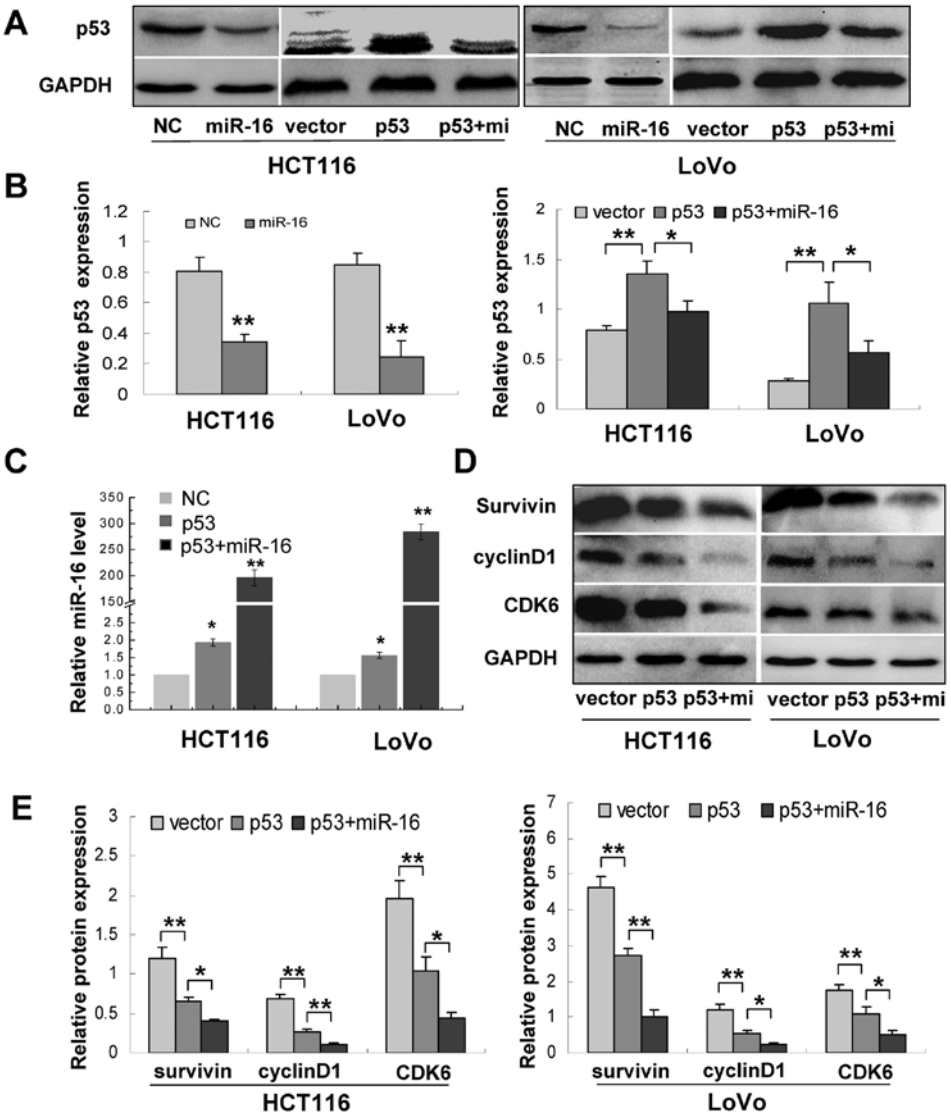

Figure 6. miR-16 regulates the p53/survivin signaling pathway. (A) Expression of p53 was examined by western blot assays in HCT116 and LoVo cells transfected with scrambled control (NC), miR-16 mimics, control vectors, the p53 plasmid and p53 plasmid+miR-16 mimics as described in Materials and methods. (B) Graphical representation of the western blot analysis in A. (C) Expression of miR-16 was determined by real-time RT-PCR in HCT116 and LoVo cells 48 h after transfection with the control vector, the p53 plasmid and the p53 plasmid+miR-16 mimics. (D) Expression of survivin, cyclin D1 and CDK6 was examined by western blot assays in HCT116 and LoVo cells transfected with the control vector, the p53 plasmid and the p53 plasmid+miR-16 mimics. (E) Graphical representation of the western blot analysis in D. The data shown are representative of 3 independent experiments. $^{**}P<0.01$.

compared to cells transfected with p53 alone (Fig. 6A and B). Then, we transfected HCT116 and LoVo cells with plasmids expressing wild-type p53. Our PCR assays showed that, compared with cells transfected with the control vector, p53 overexpression caused a 2- and 1.5-fold increase in the levels of miR-16 in HCT116 and LoVo cells, respectively (Fig. 6A-C). Meanwhile, the levels of several miR-16 targets cyclin D1, CDK6 and survivin were also reduced in cells treated with the wild-type p53 plasmid, whereas miR-16 and p53 cotransfection exerted stronger inhibition of these targets (Fig. 6D and E). Taken together, these results indicate that miR-16 regulates the p53/survivin signaling pathway.

Discussion

Deregulation of miR-16 has been demonstrated in several tumors. miR-16 has been described as a tumor suppressor and is consistently downregulated in CLL, non-small cell lung cancer and pituitary adenomas (4,8,15). However, the association between p53, miR-16 and survivin remains largely unknown. Our results clearly showed that miR-16 was downregulated in CRC tissues and was correlated with the degree of histological differentiation, which was a stage-independent prognostic factor in CRC patients (12). We further demonstrated that miR-16 inhibited proliferation and induced apoptosis of CRC cells through the intrinsic apoptosis pathway. Survivin is undetectable in normal, differentiated adult tissues, but is abundantly expressed in cancer tissues (16). Shen *et al* constructed an oncolytic adenovirus with a survivin-targeted small hairpin and found that adenovirus mediated survivin knockdown and suppressed human colorectal carcinoma growth *in vitro* and *in vivo* (17). In the present study, we found that survivin was a direct target of miR-16. Moreover, miR-16 downregulated p53 expression while p53 upregulated miR-16

levels, suggesting the presence of a regulatory loop between the two molecules. p53 also concomitantly inhibited the expression of cyclin D1, CDK6 and survivin, indicating that p53 regulates cyclin D1, CDK6 and survivin through miR-16.

Survivin is a member of the inhibitor of apoptosis *(IAP)* gene family, which was discovered in 1997. It is implicated in multiple essential functions, including cell division, checkpoint mechanisms of genomic integrity and apoptosis (18). Survivin also plays an important role in transition from adenoma with low dysplasia to high dysplasia during human colorectal tumorigenesis (19). Our data indicated that miR-16 regulated survivin expression at both the mRNA and protein levels; and miR-16 expression inhibited survivin expression and induced apoptosis in CRC cells. Additionally, expression of miR-16 in HCT116 cells repressed 3'-UTR reporter activity of vectors expressing the wild-type miR-16 binding site in the 3'-UTR of survivin, but not that of vectors expressing the mutated seed sequence. These results confirmed that survivin was one of the direct targets of miR-16.

Survivin expression was also reported to correlate with the p53 status (20). Mutant *p53* increases while wild-type *p53* represses the expression of survivin (21). Wild-type *p53* transcriptionally repressed survivin by binding to its promoter (22), histone deacetylases and methylation of its promoter (23-25). However, another study did not find that p53 could physically associate with the *survivin* promoter (24). Studies have indicated that survivin inhibited by p53 is not correlated with methylation of the survivin promoter (25,26). Therefore, the mechanisms whereby p53 represses survivin are still unclear due to the complexity of its regulation. In our study, we first showed that p53 repressed survivin expression by upregulating miR-16 in CRC cells. The relationship between p53 and miR-16 is similar to that observed in this study where p53 upregulates miR-145 (27). In other words, p53 possibly regulates survivin through the miR-16 pathway in CRC cells. Taken together, miR-16 and their regulators comprise an intricate network that could be intimately implicated in oncogenesis and tumor progression. In summary, our results demonstrated that miR-16 was downregulated and inversely related to histological differentiation in CRC tissues, and overexpression of miR-16 inhibited proliferation and induced apoptosis of CRC cells. Furthermore, we showed that survivin was a direct target of miR-16 and miR-16 regulated the p53/survivin signaling pathway. Our data suggest that miR-16 may be an important tumor suppressor in CRC cells.

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