MicroRNA-30b functions as a tumour suppressor in human colorectal cancer by targeting KRAS, PIK3CD and BCL2

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Abstract

Colorectal cancer (CRC) is the third most common cancer in the USA. MicroRNAs play important roles in the pathogenesis of CRC. In this study, we investigated the role of miR-30b in CRC and found that its expression was significantly lower in CRC tissues than that in normal tissues. We showed that a low expression level of miR-30b was closely related to poor differentiation, advanced TNM stage and poor prognosis of CRC. Further experiments showed that over-expression of miR-30b suppressed CRC cell proliferation in vitro and tumour growth in vivo. Specifically, miR-30b promoted G1 arrest and induced apoptosis. Moreover, KRAS, PIK3CD and BCL2 were identified as direct and functional targets of miR-30b. MiR-30b directly targeted the 3′-untranslated regions of their mRNAs and repressed their expression. This study revealed functional and mechanistic links between miRNA-30b and oncogene KRAS, PIK3CD and BCL2 in the pathogenesis of CRC. MiR-30b not only plays important roles in the regulation of cell proliferation and tumour growth in CRC, but is also a potential prognostic marker or therapeutic target for CRC. Restoration of miR-30b expression may represent a promising therapeutic approach for targeting malignant CRC.

Keywords: miR-30b; proliferation; apoptosis; colorectal cancer; KRAS; PIK3CD; BCL2

Introduction

CRC, one of the most common types of malignant tumour, shows high morbidity and mortality [1]. The initiation of CRC is a complicated network with multiple steps and multiple molecular processes. In the classic adenoma–carcinoma model of colorectal tumourigenesis, CRC is initiated by progressive accumulation of genetic alterations that lead to the malignant transformation from normal colorectal epithelial cells to adenocarcinoma cells [2]. Some of the key issues in CRC initiation include activation of the canonical Wnt pathway, mutational activation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS), oncogenic activation of the phosphatidylinositol 3-kinase (PI3K) pathway and inactivation of TP53 [3–5]. Activation of KRAS signalling promotes tumourigenesis through stimulation of various downstream signal pathways, including extracellular signal-regulated kinase (ERK), PI3K/Akt and NF-κB [6,7].

Recent studies have reported that microRNAs (miRNAs) are novel biomarkers of CRC [8]. Dysregulation of miRNAs has a functional role in the development and progression of CRC via post-transcriptional regulation of the expression of target mRNA transcripts by binding to their 3′-untranslated region (UTR) [9,10]. For example, altered expressions of miR-21, miR-31, miR-143 and miR-145 are closely related to clinicopathological features of CRC [11]. MiR-21 down-regulates tumour suppressor Pdcd4 and PTEN at the post-transcriptional level and regulates cell proliferation and invasion in CRC [12,13]. MiR-103/107 is over-expressed in CRC and its over-expression promotes metastasis of CRC by targeting the metastasis suppressors DAPK and KLF4 [14]. However, the role of miRNAs in the regulation of key genes and signalling pathways associated with colorectal tumourigenesis remains largely unknown. Therefore, further research is required to identify miRNAs that are involved in the development and progression of CRC.

MiR-30b is a member of the miR-30 family, which may function as important regulators in human cancer [15–20]. Several global miRNA expression analyses have showed that miR-30b is down-regulated in human CRC [21–25]. However, the clinicalpathological correlations and biological functions of miR-30b in the control of colorectal tumourigenesis have not been characterized. We set out to explore these issues.
Materials and methods

Tissue specimens and cell cultures

For the use of clinical materials for research purposes, prior approval was obtained from the Southern Medical University Institutional Board (Guangzhou, China). All samples were collected and analysed with the prior written, informed consent of the patients. CRC tissue samples \((n = 91)\) were collected between 2006 and 2008 at the Department of Pathology, Southern Medical University, and 40 CRC tissues and the matched adjacent normal tissues were obtained between 2011 and 2012. All the tissue biopsies used here were freshly frozen in liquid nitrogen and stored at \(-80^\circ\)C until further use. The medical records of the patients were reviewed to collect the following clinicopathological information: age, gender, differentiation and TNM stage. Survival data were available for the cohort of 91 patients. The median follow-up time was 55 (range 6–72) months.

Human CRC cell lines SW480, DLD1, HCT116, HCT15, KM12 and Ls174t were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria); SW620 and HT29 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (PAA).

RNA extraction and real-time PCR

For miRNA quantification, total miRNA was extracted from the cells and tissues using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. The cDNA was then synthesized from total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system, using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) containing 5 ng cDNA and 10 pM of each primer. The cycling conditions were: one cycle at 94°C for 5 min; 40 cycles of 95°C for 30 s, 56°C for 30 s. Melting curve analysis was carried out for each PCR reaction to confirm the specificity of amplification. The expression of miRNA was calculated based on the threshold cycle \((C_T)\), and relative expression levels were calculated as \(2^{-\Delta\Delta C_T}\) \((\Delta C_T = C_T \text{ of miR-30b} - C_T \text{ of U6})\) after normalization with reference to the quantification of U6 small nuclear RNA expression. Real-time PCR for target genes was performed as previously described [26]. The primers used are shown in Table S1 (see supplementary material).

Plasmids and transfection

To generate a miR-30b expression vector, a 499 bp genomic fragment covering the region encoding primary miR-30b and its up- and downstream region was PCR-amplified and cloned into the pLVthm vector (Addgene Inc., Cambridge, MA, USA). The full-length KRAS 3′ UTR is 4686 bp, the PIK3CD 3′ UTR 2068 bp and the BCL2 3′ UTR 5279 bp. The miR-30b binding site in the KRAS 3′ UTR is located at 4368–4374 bp, at 137–144 bp in the PIK3CD 3′ UTR and at 4002–4008 bp in the BCL2 3′ UTR. The region of the human KRAS 3′ UTR at 4139–4603 bp, the PIK3CD 3′ UTR at 37–276 bp and the BCL2 3′ UTR at 3831–4160 bp were generated by PCR amplification and subcloned into the BamHI/SalI sites of the pGL3–basic luciferase reporter plasmid (Promega). The primers used to generate these constructs are listed in Table S2 (see supplementary material). The miR-30b mimics, negative control and anti-miR-30b inhibitors were purchased from Genecopoeia (Guangzhou, Guangdong, China) and transfected into CRC cells using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Two concentrations of miR-30b mimics or anti-miR-30b inhibitors (10 and 50 nM) were applied.

Western blotting

Protein lysates were prepared, subjected to SDS–PAGE, transferred onto PVDF membranes and blotted according to standard methods, using anti-KRAS (Proteintech, USA), anti-PIK3CD (Santa Cruz, CA, USA), anti-Ki-67 (Abcam, Cambridge, MA, USA), anti-p27, anti-p21, anti-CyclinD1, anti-BCL2, anti-P-ERK1/2, anti-RAF1 (Bioworld Technology, Danvers, MA, USA), anti-pERK1/2, anti-BAD and anti-P-BAD antibodies (Cell Signaling Technology, Danvers, MA, USA). An anti-α-tubulin (Sigma, St. Louis, MO, USA) monoclonal antibody was used as a loading control.

MTT assay, colony formation assay, soft-agar colony formation assay, flow cytometry and luciferase assays

The miR-30b mimics, anti-miR-30b inhibitors and negative control oligos were transiently transfected into CRC cells for the MTT assay, colony-formation assay, soft agar colony-formation assay, flow-cytometry and luciferase assays, as previously described [27]. Further details are provided in Supplementary materials and methods (see supplementary material).

Terminal transferase dUTP nick end labelling (TUNEL) assay

The TUNEL assay was used to detect DNA degradation of nuclear chromatin in apoptotic cells, according to the manufacturer’s instructions (Promega, USA). In brief, \(5 \times 10^4\) cells were seeded on glass coverslips in 24-well plates and cultured for 24 h. Then these cells were treated with doxorubicin (1.0 µM) for 12 h and miR-30b mimics or miR-30b inhibitor oligos were transiently transfected into these cells. After transfection for 24 h, the cells were fixed by paraffin for 1 h,
followed by 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 min at room temperature. After washing with PBS for 3 min, the slides were incubated with 20 µg/ml proteinase K for 20 min at room temperature. The slides were then washed with 1 × PBS and incubated with 1 × TdT buffer for approximately 30 min at room temperature. Subsequently, the slides were incubated with the mixture of 57 µl binding buffer and 3 µl TdT enzyme for 60 min at 37 °C. After being washed three times with PBS, a fluorescence microscope was used to analyse the prepared cells.

Xenograft model in nude mice
For tumourigenesis assays, we engineered SW620 cells to stably over-express miR-30b and SW480 cells to stably express low miR-30b, using a lentiviral-based system (pLVTHM).

Xenograft tumours were generated by subcutaneous injection of CRC cells (2 × 10⁶), including SW620/Vector, SW620/miR-30b, SW480/NC and SW480/miR-30b inhibitor, into the hind limbs of 4–6-week-old Balb/C athymic nude mice (nu/nu; Animal Centre of Southern Medical University, Guangzhou, China; n = 6 for each group). All mice were housed and maintained under specific pathogen-free conditions, and all experiments were approved by the Use Committee for Animal Care and performed in accordance with institutional guidelines. Tumour size was measured using a slide caliper and tumour volume was determined by the formula: \( V = \frac{1}{2} \times A \times B^2 \), where \( A \) represents the diameter of the base of the tumour and \( B \) represents the corresponding perpendicular value. After euthanasia, the tumours were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, and 4 µm sections were prepared and stained with haematoxylin.

Statistical analysis
All statistical analyses were performed using SPSS19.0 for Windows. The two-tailed paired Student’s \( t \)-test was used for analysing two groups. The Mann–Whitney U-test and Spearman’s correlation analyses were used to analyse the relationship between miR-30b expression and the clinicopathological features of CRC. Survival curves were plotted by the Kaplan–Meier method and compared with the log-rank test. \( p < 0.05 \) was considered statistically significant.

Results
Down-regulation of miR-30b correlates with aggressive characteristics of CRC and poor survival
We first tested the expression of miR-30b by real-time PCR analysis in 40 CRC biopsies. The result revealed that miR-30b was down-regulated in 80% (32/40) of the CRC tissue samples examined (N/T > two-fold) compared to their matched adjacent normal tissues, with up to 20-fold decreases observed in the CRC tissue samples (see supplementary material, Figure S1A). Student’s \( t \)-test revealed that the miR-30b expression levels were significantly lower in the CRC tissues samples than in adjacent normal tissues (\( p < 0.001 \); see supplementary material, Figure S1B).

To further investigate the clinicopathological significance of miR-30b, the levels of miR-30b were quantified in six normal intestine epithelial tissues and a cohort of 91 cases of archived CRC samples, using real-time PCR. As shown in Figure 1, relative miR-30b expression levels were significantly lower those that in control normal tissues (Figure 1A) and decreased along with the progression of T classifications (Figure 1B), N classifications (Figure 1C), distant metastasis (Figure 1D) and poor differentiation (Figure 1E). The median relative expression level (3.15) of miR-30b in all 91 CRC samples was chosen as the cut-off point for separating tumours with low expression (< median, \( n = 46 \)) and high expression (≥ median, \( n = 45 \)) of miR-30b. Mann–Whitney U-tests (Table 1) and Spearman’s correlation (see supplementary material, Table S3) analysis showed that low expression of miR-30b was positively associated with poor differentiation and advanced TNM stage. Kaplan–Meier survival analysis and log-rank tests revealed that patients with high miR-30b expression had longer survival times, whereas those with low miR-30b expression had shorter survival times (Figure 1F, log-rank, \( p = 0.01 \)). Univariate and multivariate analyses of various prognosis parameters showed that miR-30b expression level was an independent prognostic factor for outcomes in patients with CRC (see supplementary material, Table S4).

Ectopic expression of miR-30b inhibits cell proliferation and tumour growth ability of CRC cells
MiR-30b expression was measured in eight CRC cell lines by real-time PCR analysis. The results showed that miR-30b expression was relatively lower in SW620 and HCT116 than that in SW480 and HCT15 (Figure 2A). Therefore, to evaluate the possible role of miR-30b in CRC pathogenesis, we over-expressed miR-30b in SW620 and HCT116 cells by transfecting hsa-miR-30b mimic oligonucleotides (Figure 2B). MTT and colony-formation assays revealed that overexpression of miR-30b significantly decreased the growth rate of SW620 and HCT116 cells compared with Vector-transfected cells (Figure 2C, D). Additionally, the proliferation index (calculated by Ki-67 expression) was dramatically decreased in miR-30b-over-expressing cells compared with that of Vector-transfected cells (Figure 2E).

We next examined the effect of miR-30b on the anchorage-independent growth ability of CRC cells, using the soft agar assay. Over-expression of miR-30b significantly reduced the growth of SW620 and HCT116 cells (Figure 2F; \( p < 0.01 \)). To confirm this
Figure 1. Expression of miR-30b in CRC tissues and its clinical significance. MiR-30b expression was assessed by real-time PCR and was normalized by U6 expression. The bounds of boxes represent the lower and upper quartiles; lines within boxes and whiskers denote median and extremum, respectively. (A) Mean expression of miR-30b in the 91 human CRC tissues and six normal intestine epithelial tissues. (B) Correlation between miR-30b expression and T classification (T1–T4) in 91 cases of CRC tissues and normal intestine epithelial tissues (normal, n = 6). (C) Correlation between miR-30b expression and N classification of CRC (N0–N2). (D) Correlation between miR-30b expression and distant metastasis. (E) Correlation between miR-30b expression and differentiation of CRC. (F) Correlation between miR-30b levels and survival by Kaplan–Meier analysis of patients with high (≥ median; n = 45) or low miR-30b (< median; n = 46) expression; median = 3.15; **p < 0.01

Table 1. Correlation between clinicopathological features and miR-30b expression in 91 CRC tissues

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Inhibition of endogenous miR-30b promotes cell proliferation and tumour growth ability of CRC cells

We next suppressed miR-30b in SW480 and HCT15 by expressing miR-30b inhibitors (Figure 3A). Suppression of miR-30b significantly increased the growth rate of SW480 and HCT15 cells compared with negative control (NC)-transfected cells (Figure 3B, C). Additionally, the proliferation index was significantly increased in miR-30b-suppressing cells compared with that of NC-transfected cells (Figure 3D). Moreover, depletion of endogenous miR-30b caused a significant increase in colony number and size in soft agar (Figure 3E; p < 0.01). In order to observe the effects of miR-30b inhibition on tumour growth in nude mice, we used a lentiviral-based system and established stable low miR-30b-expressing SW480 cells. As shown in Figure 3F, the tumours in the SW480-miR-30b

Table 1. Correlation between clinicopathological features and miR-30b expression in 91 CRC tissues

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miR-30b target genes in CRC

Figure 2. Over-expression of miR-30b inhibits proliferation and tumour growth of CRC cells. (A) miR-30b expression assessed by real-time PCR in eight CRC cell lines. (B) Ectopic expression of miR-30b in SW620 and HCT116 CRC cells was validated by real-time PCR. (C) Cell growth analysed by MTT assays. (D) Representative results of colony formation; the numbers of colonies containing 50 cells were scored. The number of colonies counted was of an entire well and the error bars represent mean ± SD from three independent experiments. (E) Quantification of the numbers of Ki-67-positive cells (yellow-brown) by immunohistochemical staining. (F) Anchorage-independent colony-formation assays; only cell colonies > 0.1 mm in diameter were counted. (G) SW620-Vector and SW620-miR-30b cells were injected into the hind limbs of nude mice (n = 6). Tumour volumes were measured on the indicated days. Data points are presented as the mean tumour volume ± SD. (H) Real-time PCR analysis of miR-30b expression in tissues of resected tumours formed from SW620-Vector and SW620-miR-30b. (I) Histopathological analyses of xenograft tumours. The tumour sections were stained with H&E or subjected to IHC staining using an antibody against Ki-67. Error bars represent mean ± SD from three independent experiments.

inhibitor group grew much faster than those in the SW480-NC group. The expression of miR-30b was significantly lower in the SW480-miR-30b inhibitor group than in the SW480-NC group (Figure 3G). Furthermore, IHC confirmed that tumours of the miR-30b-inhibitor displayed much higher Ki-67 indices than tumours from the control group (Figure 3H).

MiR-30b prohibits cell cycle progression and promotes doxorubicin-induced apoptosis in CRC cells

To explore the possible mechanism of miR-30b’s function in controlling CRC cell proliferation and tumour growth, we determined the distribution of
cells within the stages of the cell cycle by flow cytometry. Cells treated with miR-30b mimics showed a significant increase in the percentage of cells in the G1/G0 peak and a decrease in the percentage of cells in the S and G2/M peak (p < 0.01) (Figure 4A). However, cells treated with miR-30b inhibitor showed a dramatic decrease in the percentage of cells in the G1/G0 peak and an increase in the percentage of cells in the S and G2/M peak (p < 0.01) (Figure 4B). These results suggested that miR-30b could inhibit cell proliferation by arresting the tumour cells at G1/G0 phase.

We next measured the effect of miR-30b on apoptosis of CRC cells. The CRC cells were treated by doxorubicin (1.0 µM) for 12 h, and miR-30b mimics and miR-30b-inhibitor were transfected into these cells. After transfection for 24 h, the apoptosis rate was analysed by flow cytometry. The results showed that the rate of apoptosis was significantly higher in SW620 and HCT116 cells over-expressing miR-30b (Figure 4C). However, the rate of apoptosis was markedly decreased when miR-30b was inhibited in SW480 and HCT15 cells (Figure 4D). In addition, TUNEL assays confirmed that more apoptotic cells could be observed in SW620 and HCT116 cells treated with miR-30b mimics than in control cells (Figure 4E), while fewer apoptotic cells were detected in SW480 and HCT15 cells treated with the miR-30b inhibitor than in NC-transfected cells (Figure 4F).
miR-30b target genes in CRC

Figure 4. MiR-30b prohibits cell cycle progression and promotes doxorubicin-induced apoptosis in CRC cells. (A, B) Flow-cytometry analyses of the cell cycle of the indicated CRC cells synchronized in the G2/M phase by treatment with 0.1 µM colchicine for 12 h. (C, D) Apoptosis assay by flow cytometry: (left panel) flow-cytometry analyses of various CRC cells treated with 1.0 µM doxorubicin for 24 h; (right panel) quantification of apoptotic rate. Annexin-positive/PI-negative (right lower quadrant) cells were analysed for apoptosis rate. Error bars represent mean ± SD from three independent experiments. (E, F) Apoptosis assay by terminal transferase dUTP nick end labelling (TUNEL): (left panel) TUNEL staining of various CRC cells treated with 1.0 µM doxorubicin for 24 h; (right panel) quantification of TUNEL-stained cells. TUNEL staining-positive cells were counted in 10 random fields after the indicated cells had been treated with doxorubicin (1.0 µM) for 24 h. Apoptotic cells are stained in green in the nucleus.

**KRAS, PIK3CD and BCL2 are direct targets of miR-30b**

Three publicly available bioinformatic algorithms (TargetScan, Pictar, miRANDA) were used to analyse target genes of miR-30b, and GO analysis and KEGG pathway analysis was performed (see supplementary material, Tables S5, S6). The results showed that **KRAS, PIK3CD and BCL2** were theoretical target genes of miR-30b (Figure 5A). Western blot analyses showed that the protein levels of KRAS, PIK3CD and BCL2 were dramatically down-regulated in miR-30b-over-expressing cells, whereas they were up-regulated after inhibition of miR-30b (Figure 5B, C).
Figure 5. KRAS, PIK3CD and BCL2 are direct targets of miR-30b. (A) Predicted miR-30b target sequences in the 3′ UTRs of KRAS, PIK3CD and BCL2, and their mutants containing altered nucleotides in the 3′ UTRs. (B) Western blot analysis of KRAS, PIK3CD and BCL2, and the protein products of their downstream genes in the indicated cells. (C) Real-time PCR analysis of KRAS, PIK3CD and BCL2, and the mRNA expression of their downstream genes. (D–F) Luciferase assay analyses of the indicated cells transfected with the indicated reporters with increasing amounts of miR-30b (10 and 50 nM). Error bars represent mean ± SD from three independent experiments; **p < 0.01.

We subcloned the KRAS, PIK3CD and BCL2 3′ UTR fragments containing one miR-30b binding site and their mutant fragments into the pGL3–basic luciferase reporter vectors. As shown in Figure 5D–F, a consistent and dose-dependent reduction of luciferase activity was observed upon miR-30b transfection in both CRC lines, but mutations in the tentative miR-30b-binding seed region in KRAS, PIK3CD and BCL2 3′ UTRs abrogated the suppressive effect on KRAS, PIK3CD and BCL2 mediated by miR-30b. These results demonstrated that miR-30b could directly target KRAS, PIK3CD and BCL2 in CRC cells by interaction with the 3′ UTRs of these genes. The direct targeting of KRAS and PIK3CD by miR-30b suggested that the activity of the KRAS and PI3K signalling pathway could be modulated. Therefore, the expressions of genes downstream of KRAS and PI3K in the signalling pathway were assayed. The results showed that the expression of p-RAF1, p-ERK1/2, p-AKT and p-BAD and were significantly decreased
Repression of KRAS, PIK3CD and BCL2 plays crucial roles in miR-30b-induced cell cycle arrest and apoptosis of CRC cells

We next restored the expression of KRAS, PIK3CD and BCL2 in miR-30b-over-expressing SW620 cells (Figure 6A) by transfection of KRAS, PIK3CD and BCL2 ORF constructs without 3′ UTRs, and observed their effects on proliferation and apoptosis. The results showed that single-gene re-expression of KRAS, PIK3CD or BCL2 increased the growth rate of SW620 cell mildly; however, the co-expression of KRAS, PIK3CD and BCL2 remarkably enhanced the cells’ growth rate (Figure 6B). Moreover, the expression of KRAS alone did not significantly inhibit apoptosis, and the expression of PIK3CD or BCL2 alone caused a modest decline in apoptosis, whereas the co-expression of KRAS and PIK3CD significantly inhibited apoptosis in CRC cells (Figure 6C).

To further examine whether the findings above could be supported by observations in human primary tumours, the correlation between miR-30b expression and the expression of KRAS, PIK3CD or BCL2 was analysed in 10 freshly collected CRC biopsies (Figure 6D; see also supplementary material, Figure S2). Figure 6E shows that miR-30b expression negatively correlated with the expressions of KRAS (\(r = -0.881, p < 0.001\)), PIK3CD (\(r = -0.786, p < 0.001\)) and BCL2 (\(r = -0.852, p < 0.001\)). Moreover, the expression of KRAS, PIK3CD and BCL2 in xenograft tumours formed by SW620/Vector cells and SW620/miR-30b cells were measured by IHC, which demonstrated that the KRAS, PIK3CD and BCL2 proteins were expressed at lower levels in the tumours generated from miR-30b-over-expressing cells, compared with the vector control cells (see supplementary material, Figure S3).

**Discussion**

MiRNAs are a class of regulatory molecules that regulate the expression of target genes by binding to specific sites of their mRNAs [28]. A crucial role for miRNAs in tumour development has been suggested by the identification of numerous miRNAs dysregulated in many types of tumour [29,30]. Emerging research suggests that miRNAs play essential roles in the progression of CRC and CRC therapies [31]. Several global miRNA expression analyses have revealed that miR-30b is down-regulated in human CRC [21–25].

However, no further expression or functional confirmation of miR-30b in CRC has been reported. Here, we reported that miR-30b was down-regulated in human CRC tissues. MiR-30b over-expression was able to induce growth inhibition in CRC cancer cells, which was accompanied by an increase of cells arrested in the G1 phase and by an induction of cellular apoptosis. Moreover, miR-30b suppressed the expression of KRAS, PIK3CD and BCL2 by directly targeting the 3′ UTRs of their mRNAs. This suggests a mechanistic connection between miR-30b dysregulation and the pathogenesis of human CRC, and may provide new insights for CRC research and therapeutic strategies for CRC prevention and treatment.

It is believed that miRNAs function as tumour suppressors or oncogenes in the genesis of a variety of cancers, including CRC [32]. For example, miR-200 family members are important tumour suppressors [33], while miR-21 represents an onco-microRNA in CRC [13]. The miR-30 family comprises miR-30a, -30b, -30c, -30d, -30e and -384-5p. Recent reports provided evidence that miR-30 members may function as tumour suppressors [15–18] or onco-miRNAs [19,20]. For instance, MiR-30a suppresses cell migration, invasion and tumour growth in CRC [15,16]. MiR-30c inhibits proliferation of breast cancer cells [17]. MiR-384-5p induces apoptosis and suppresses cell viability [18]. Conversely, miR-30e can promote invasiveness and metastasis in glioma cells [20].

There are two opposite views of miR-30b’s function in human cancer. It is amplified and up-regulated in oral squamous cell cancers, medulloblastoma and melanoma [34–36]. However, in other studies, miR-30b was considered a tumour-suppressor miRNA [37]. The expression of miR-30b was suppressed in CRC [24], invasive bladder cancer [38] and lung squamous cell carcinoma [39]. Ectopic expression of miR-30b suppresses cell growth [37] and reduces tumourigenesis and lung metastasis in non-obese diabetic/severe combined immunodeficient mice [34]. Our results indicated that miR-30b was down-regulated in human CRC, could induce G1 arrest and apoptosis and inhibit tumour growth. Thus, our data support the view that miR-30b mainly functions as a tumour suppresser in CRC.

Recently, it was reported that miR-30b/c resist TRAIL-induced apoptosis in glioma cells [40]. These converse effects of miR-30b on apoptosis in different cells may depend on the cellular context and the target genes. Most glioma cells have been reported to be resistant to TRAIL-induced apoptosis [41]. However, over-expression of BCL2 [42,43] and KRAS [44,45] mutations are more frequent events in CRC. Thus, it is possible that miR-30b promotes resistance to apoptosis in glioma, while promoting apoptosis in CRC cells. This cell type-specific function has been observed with other miRNAs, such as miR-221/222, which are over-expressed in the majority of epithelial tumours [46] but play a tumour-suppressive role in erythroleukaemic cells by inhibiting erythropoiesis through the down-regulation of c-Kit receptor [47].
Figure 6. Repression of KRAS, PIK3CD and BCL2 plays a crucial role in miR-30b-induced cell cycle arrest and apoptosis of CRC cells. (A) Western blot analysis and real-time PCR of KRAS, PIK3CD and BCL2 ectopic expression in SW620 cells. (B) Cell growth of the indicated cells analysed using MTT. The OD values (450 nm) of cells at day 7 were analysed; error bars represent mean ± SD from three independent experiments; **p < 0.01. (C) Apoptosis of the indicated cells analysed using flow cytometry; error bars represent mean ± SD from three independent experiments; **p < 0.01. (D) Real-time PCR analysis of miR-30b, KRAS, PIK3CD and BCL2 expression (upper panel) and western blot analysis of KRAS, PIK3CD and BCL2 in the 10 fresh human CRC samples; α-tubulin was used as loading control (lower panel). (E) Spearman correlation analyses between relative miR-30b expression and relative mRNA expression levels of KRAS, PIK3CD and BCL2 in 10 fresh human CRC samples.

Interestingly, miR-30b maps to 8q24, a region contains the c-Myc gene, and is often amplified in many cancers, including CRC [48,49]. This phenomenon seems to be contradictory to our results. In the past few years, examples of structural variation in the human genome, including amplification and deletion, have been uncovered. There are regions that contain all these types of variation. An example is 8p23.1, which is often deleted in heart disease but frequently amplified in oesophageal cancer [50]. In addition, the distance
between these two regions is > 7 Mb. Thus, although the region that contains c-Myc is often amplified, it could not be excluded that the region containing miR-30b is deleted in CRC. Moreover, the expression level is not always positively correlated with the genomic copy number [50, 51]. The expression of miRNAs can be regulated at different stages, including amplification, transcriptional and post-transcriptional levels, and epigenetic changes [52]. Therefore, the mechanism that leads to the down-regulation of miR-30b in CRC needs further investigation.

The KRAS gene is mutated in nearly 50% of CRCs at an early stage of tumourigenesis, playing essential roles in cell growth, survival, proliferation and even metastasis in CRC [53]. The key downstream pathways of activated KRAS are the RAS–Raf–MEK–ERK and RAS–PI3K–PTEN–Akt signalling pathways, which are prime therapeutic targets for diverse human cancers [54]. The effects of Ras–Raf–MEK–ERK and Ras–PI3K–PTEN–Akt pathways on proliferation and apoptosis are mainly mediated by ERK or Akt regulation of key cell cycle regulators or apoptotic effectors, such as CyclinD1, BCL2, Bad, Bim, FoxO and Caspase-9 [55]. PIK3CD is an important component of the PI3K–PTEN–Akt pathway and plays important roles in proliferation and survival [56]. In this study, we found that miR-30b could inactivate the Raf–MEK–ERK and PI3K–PTEN–Akt pathways, resulting in inhibition of BCL2, p-BAD expression, down-regulation of CyclinD1 and up-regulation of p27. Importantly, we identified a dual inhibition of KRAS-induced activation of the Raf–MEK–ERK and PI3K–PTEN–Akt signalling pathways by miR-30b, through directly targeting KRAS and PIK3CD by binding to the 3′ UTR of their mRNAs. The anti-apoptotic molecule BCL2 is an essential regulator of the intrinsic apoptotic pathway, and has been implicated in the development and progression of CRC [57]. BCL2 is also a downstream effector of ERK or AKT [58]. The present study reveals that miR-30b inhibits BCL2 expression, indirectly through regulation of RAS–Raf–MEK–ERK and RAS–PI3K–PTEN–Akt and directly by binding to the 3′ UTR of BCL2 mRNA.

Taken together, our findings suggest that miR-30b is down-regulated in CRC, and may affect tumour cell survival in CRC by inhibiting three targets: KRAS, PI3KCD and BCL2. Restoration of miR-30b might represent a useful therapeutic approach for targeting malignant CRC.

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Author contributions

WTL and YQD designed the experiments; YPY, NJZ, TTL and SYW conducted experiments; LQ, YMC, PW and JHL provided research materials and methods; YJX, CZ and JXW analysed data; and WTL and YPY wrote the manuscript.

References


SUPPLEMENTARY MATERIAL
The following supplementary material may be found in the online version of this article:

Supplementary materials and methods
Figure S1. Expression of miR-30b in CRC tissues
Figure S2. Immunohistochemical analyses of KRAS, PIK3CD and BCL2 expression in primary human CRC specimens with low or high miR-30b expression
Figure S3. Expression of KRAS, PIK3CD and BCL2 in xenograft tumours
Table S1. Primer sequences used for real-time PCR (5' to 3')
Table S2. Primer sequences used for amplification and plasmid construction (5' to 3')
Table S3. Spearman correlation analysis between relative miR-30b expression and clinicopathological features
Table S4. Univariate and multivariate analyses of various prognosis parameters in 91 CRC patients using Cox regression model
Table S5. GO analysis of miR-30b's target genes
Table S6. KEGG pathway analysis of miR-30b's target genes

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