MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5

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Abstract

One major reason for the failure of advanced colorectal cancer (CRC) treatment is the occurrence of chemoresistance to fluoropyrimidine (FU)-based chemotherapy. Various reports showed that ectopic expression and function of microRNAs (miRNAs) played key roles to mediate apoptosis at the post-transcriptional level. To further explore the possible mechanisms, we evaluated the prognostic effect of miR-218 in patients with CRC receiving 5-FU-based treatment and investigated the proapoptotic role of miR-218 in vitro. Primary tumour specimens and adjacent non-tumour sites were used to determine miR-218 expression distribution and explore its potential prognostic value in response to 5-FU-based treatment in patients with CRC. HCT116 and HT29 cells were transfected with precursor miR-218 or negative control, followed by assays to investigate its influence on apoptosis, cell proliferation and pathways involved in molecular mechanisms of chemoresistance to 5-FU. Results showed that high miR-218 expression was associated with positive response to firstline 5-FU treatment in CRC patients. MiR-218 promoted apoptosis, inhibited cell proliferation and caused cell cycle arrest in CRC cells by suppressing BIRC5 expression. Furthermore, miR-218 enhanced 5-FU cytotoxicity in CRC cells by suppressing the 5-FU targeted enzyme, thymidylate synthase (TS). In conclusion, we demonstrated that high miR-218 expression had a positive prognostic value in 5-FU-based treatments for CRC patients and discovered a novel mechanism mediated by miR-218 to promote apoptosis and to function synergistically with 5-FU to promote chemosensitivity by suppressing BIRC5 and TS in CRC. These suggest the unique potential of miR-218 as a novel candidate for developing miR-218-based therapeutic strategies in CRC.

Introduction

Colorectal cancer (CRC) is associated with high mortality, and is considered the fifth leading cause of cancer-related deaths in China (1). Although many kinds of treatments have been developed and used in patients with CRC, surgery resection with subsequent chemotherapy is still the most commonly used method, especially for patients with stages II and III disease. Currently, 5-fluorouracil (5-FU) has been commonly used for patients with CRC, and 5-FU-based chemotherapy has led to response rates of >50% and a median survival rate up to 2 years (2,3). Despite these impressive developments, a large proportion of patients with metastatic CRC finally become resistant to 5-FU, and this resistance has been a key barrier to the efficacy of CRC treatment (4).

Chemoresistance mechanisms are extraordinarily complex, including inefficient cellular drug uptake and accumulation (5), enhancement of DNA repair (6), upregulation of the
antiapoptosis pathway and activation of the antioxidant glutathione system for detoxification [7,8]. Among them, antiapoptosis has proved an important factor in the development of drug resistance, and disruptions of the apoptotic pathway have been demonstrated to increase cell proliferation and promote drug resistance [9–11]. Thus, it is essential to better understand the mechanisms of apoptotic pathway regulation and find novel strategies to improve the chemosensitivity to commonly used drugs, such as 5-FU.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that function primarily to regulate protein expression at post-transcriptional level by specifically binding to the 3′-untranslated region (3′-UTR) of mRNAs [12,13]. Ectopic expression of miRNAs has been found in many types of cancers [14,15]. In recent years, efforts have been devoted to understanding the molecular mechanisms of apoptosis, and research has revealed that miRNA post-transcriptional and translational control of gene expression had key roles in the mechanisms of apoptosis and drug resistance [16–18]. MiR-218 belongs to the SULT gene family and commonly functions as a tumour suppressor gene [19,20]. Previous studies demonstrated that miR-218 was significantly downregulated in cancer patients and had a role in cancer progression [20–22]. The identification and characterisation of the role of miR-218 in CRC may provide new insight into understanding of the molecular mechanisms of disease development and are therefore crucial for the development of new therapy options.

BIRC5 is one of key member of the inhibitors of apoptosis gene (IAP) family, which plays a regulatory role in apoptosis through the formation of a complex with X-linked inhibitor of apoptosis (XIAP) [23]. In this study, miRanda (www.targetscan.org) and TargetScan (www.microRNA.org) predict that miR-218 targets the 3′UTR of BIRC5 mRNA, which suggest that miR-218 may play a role in the BIRC5 regulation pathway. However, the specific functional significance of miR-218 and BIRC5 in CRC chemoresistance remains unknown. In this study, we identified the prognostic value of miR-218 in CRC patients and a mechanism of direct regulation of BIRC5 by miR-218, leading to activation of the intrinsic apoptosis pathway in CRC. Moreover, we found that miR-218 suppressed the expression of the 5-FU-targeted protein thymidylate synthase (TS). Thus, overexpression of miR-218 promoted apoptosis, inhibited cell proliferation and sensitised CRC cells to 5-FU treatments.

Materials and methods

Cell culture

The human CRC cell lines HT29 and HCT116 were obtained from American Type Culture Collection (Manassas, VA). The authenticity of the cells was determined by short tandem repeat analysis technology (Cell ID™ System, Promega, Madison, WI). Cell lines were cultured with RPMI 1640 (Thermo Fisher Scientific, Wilmington, DE) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St Louis, MO) and penicillin-streptomycin (Life Technologies, Grand Island, NY) at 37°C in 5% CO2, and 95% air.

Patient samples

Paired resected surgical specimens from primary tumour and adjacent non-tumour sites were obtained from 63 CRC patients who were diagnosed histologically. The patients all received standard 5-FU-based chemotherapy after surgery at Qilu Hospital of Shandong University between 2008 and 2010. Tumour response was confirmed through computed tomography and evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria as complete response, partial response, stable disease and progressive disease. Overall survival (OS) was defined as the duration from inclusion to death of any cause, and progression-free survival (PFS) was defined as the duration from inclusion to progressive disease or death of any cause. All patients received a standard follow-up with computed tomography of the abdomen after surgery. The samples were snap-frozen and stored in liquid nitrogen. Written informed consent was obtained from all patients according to the guidelines approved by the Ethics Committee of Qilu Hospital, Shandong University.

RNA extraction

Total RNA was isolated from primary tumours or CRC cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). The extracted total RNA was eluted in 20 μl nuclease-free water and the RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific).

MiRNA transfection

Cells were plated in 24-well plates at 1 x 10⁶ per well. Forty-eight hours after plating, 50 nM of miR-218 precursor (Pre-miR-218) (Pre-miR™ miRNA Precursor, Ambion, Carlsbad, CA) was transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Negative miRNAs (Applied Biosystems, Carlsbad, CA) were also transfected as negative control.

Reverse transcription quantitative PCR (RT-qPCR) for miRNA and mRNA expression

RT-qPCR of individual miRNAs was performed using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) and SYBR Premix Ex Taq (TaKaRa). The comparative threshold cycle (Ct) method was used to calculate the relative miRNA abundance compared with the RNA U6 small nuclear 2 (RNU6B-2) expression (fold difference relative to RNU6B-2).

For BIRC5 mRNA quantification, RNA was reverse-transcribed with a random primer using a TaKaRa Reverse Transcription Kit (TaKaRa, Dalian, China). miRNA levels were determined with RT-qPCR using the SYBR Green master mix on an ABI PRISM Sequence Detection System (Applied Biosystems) with the housekeeping gene GAPDH as an internal control. The BIRC5 and GAPDH primers were as follows: forward, 5′-ATTCCGGCGTGTCCGCTTCC-3′; reverse, 5′-CGACGGGCCTTCCCTGCGG-3′; and forward, 5′-GCAACGCTCAAGGCTGAGAAC-3′; reverse, 5′-TGTTGAAAGAGCCGACGTTGGA-3′, respectively.

Flow cytometry for apoptosis assay

Twenty-four hours after transfection with Pre-miR-218 or negative control, cells were harvested and stained with annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (BD Bioscience, San Diego, CA). Apoptosis was assessed by flow cytometry (BD FACSCalibur).

TUNEL assay for apoptosis detection

After transfection, cells were fixed with 4% paraformaldehyde for 60 min, and then incubated in 0.1% Triton X-100 for 2 min on ice. Apoptotic cells were analysed using the one-step TUNEL apoptosis assay kit in accordance with the manufacturer's protocol (Beyotime, Shanghai, China). Images were captured using a Nikon fluorescence microscope with an attached CCD camera (Nikon Corporation, Tokyo, Japan).

CCK-8 assay for cell viability and proliferation

Cell viability was quantified by means of the CCK-8 kit (Beijing) after 24h transfection. After overnight incubation, freshly prepared medium with or without 5-FU was then added at a final concentration gradient of 1, 1.5, 2, 2.5, 3 and 4 μM 5-FU. After
being exposed for 24, 48, 72 and 96 h, the cells were then incubated in normal medium containing WST-8 substrate at 37°C for 2 h. Finally, cell viability was assessed measuring absorbance at 450 nm with a spectrophotometer.

**Colony formation assay**

Cells were plated in 24-well plates at 1 × 10⁴ cells per well and transfected with pre-miR-218 or negative control by using Lipofectamine 2000 (Invitrogen). Then, cells were collected and seeded (300 cells per well) in a fresh six-well plate 48 h after transfection, and maintained in RPMI 1640 containing 10% foetal bovine serum. After 14 days, cells were fixed with 4% paraformaldehyde for 10 min and then stained using crystal violet. Individual colonies (>50 cells) were manually counted.

**Cell cycle analysis**

Forty-eight hours after transfection, cells were washed with ice-cold phosphate-buffered saline and fixed with 70% ethanol at −20°C for 24 h. The fixed cells were rehydrated in phosphate-buffered saline for 15 min and then stained with 50 mg/ml PI (BD Bioscience) followed by flow cytometric analysis using a FACSCalibur instrument (BD Bioscience). The final results were analysed using ModFit software (BD Bioscience).

**Dual-luciferase reporter assay**

The putative miR-218 binding sites in the BIRC5 3′UTR were predicted by TargetScan and miRanda. Dual-luciferase reporter assay was performed using pmir-REPORT™ vectors (RiboBio, Guangzhou, China) containing wild-type BIRC5 3′UTR sequences or mutant BIRC5 3′UTR sequences. Cells (1 × 10⁴) were transiently transfected with pre-miR-218 or negative control together with wild-type BIRC5 3′UTR vector or mutant type BIRC5 3′UTR vector in a 24-well plate. Cells were harvested 48 h after transfection, and luciferase activity was analysed by the Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instructions.

**Antibodies and western blot**

The primary antibodies used for western blotting were mouse anti-human BIRC5 antibody (1:1000; Abcam, Cambridge, MA), mouse anti-human β-actin antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-human TS antibody (1:1000). HRP-conjugated (HRP) antimouse antibodies (1:5000; Bio-Rad, Hercules, CA) were used as the secondary antibodies. Seventy-two hours after transfection, total protein was extracted with RIPA buffer (Sigma-Aldrich), and the protein concentration was measured by the Bradford DC protein assay (Bio-Rad). Then, 25 μg protein from each sample was separated on 10% Bis-Tris polyacrylamide gel through electrophoresis and then blotted onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were immunostained with primary antibody at 4°C overnight and with secondary antibody at room temperature for 1 h. Immunoblots were visualised using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

**Statistical analysis**

For CRC versus normal cell lines and CRC tissue versus adjacent non-tumour tissue, miRNA expression levels and luciferase reporter assays were treated as continuous variables, and differences in mean expression were determined using Student’s t-test. Differences in cell growth curves and cell cytotoxicity curves were determined by repeated measures analysis of variance. For clinical prognostic analysis, the cut-off point was defined as the expression value resulting in the greatest area under the curve in receiver operator characteristic analysis. The survival curves of CRC patients were estimated via the Kaplan–Meier method and the difference in survival curves was estimated using log-rank testing. The Mann–Whitney U test or Kruskal–Wallis test was used for evaluating the pathological difference among clinical cohort groups. Correlation testing between tissue miR-218 and BIRC5 mRNA expression was analysed using the Spearman test. Count dates were described as frequency and examined using Fisher’s exact test. The results were considered statistically significant at P < 0.05. All statistical analyses were performed with GraphPad Prism (version 5.01) software (La Jolla, CA).

**Results**

**Downregulation of miR-218 in primary CRC tissues and cell lines**

The expression of miR-218 was significantly decreased in tumour tissues compared with paired normal tissues (P < 0.001, Figure 1A). Moreover, the CRC tissues in 68.3% (43 of 63) of cases had at least 2-fold lower expression of miR-218 (Figure 1B). MiR-218 was also downregulated in the HT29 and HCT116 cell lines compared with the microdissected normal primary colon cells from normal tissues obtained from Qilu Hospital (P < 0.0001 for both, Figure 1C). As shown in Table 1, no significant correlations were observed between miR-218 expression and clinicopathological features, such as age, sex, chemotherapy method and tumour stage. We also tested the influence of 5-FU on miR-218 expression in CRC cells, and a significantly decreased miR-218 level was found after 3-day treatment with 2 and 4 μM 5-FU (P < 0.05 for both) (Figure 1D).

**High miR-218 expression was associated with positive response to first-line 5-FU treatment in CRC patients**

To investigate the potential prognostic value of miR-218, we analysed the survival rate of patients who received first-line 5-FU-based adjuvant chemotherapy and explored the association between primary tumour miR-218 expression and chemotherapy response. The results revealed a statistically significant difference between miR-218 expression and response to chemotherapy, PFS and OS (Table 1). Patients were divided into responding (complete response + partial response) and non-responding (stable disease + progressive disease) groups according to RECIST criteria. Levels of miR-218 were significantly higher in the responding group (n = 35) than in the non-responding group (n = 28) (P < 0.001, Figure 1E). Then, receiver operator characteristic curve analysis was performed to establish the optimal cut-off value of miR-218 (6 × 10⁻³, shown in Supplementary Figure 1, available at Carcinogenesis Online) for distinguishing the responding and non-responding patients. Under these stratification criteria, patients were stratified into high (n = 34) and low (n = 29) miR-218 expression groups. The proportion of patients that responded to chemotherapy was significantly higher in the high miR-218 expression group than in the low miR-218 expression group (P < 0.001, Figure 1F).

More importantly, Kaplan–Meier survival analysis was performed to further investigate the effect of miR-218 on 5-FU treatment for CRC. The results indicated that high miR-218 expression was associated with long OS (P = 0.0002, Figure 1G) and PFS (P = 0.002, Figure 1G).

**BIRC5 is a direct target of miR-218 in CRC cells**

Given the profound downregulation of miR-218 in CRC, we sought to determine the gene targets that may account for these findings. On the basis of two major prediction software, TargetScan (http://www.targetscan.org) and miRanda (http://www.micromics.com), the putative binding sites of miR-218 in the 3′UTR of BIRC5 were predicted (Figure 2A). The expression of miR-218 and BIRC5 mRNA were analysed in 63 CRC tissues, and significantly decreased BIRC5 mRNA expression was found with the increased expression of miR-218 (r = −0.89, P < 0.01, Figure 2B). To experimentally demonstrate that BIRC5 is targeted by miR-218, we transfected HT29 and HCT116 cell lines with either miR-218 precursor or negative control (Figure 2C), and quantified the mRNA and protein expression of BIRC5 using
RT-qPCR and western blotting. The results indicated that BIRC5 mRNA and protein expression was significantly reduced in cell lines transfected with miR-218 when compared with negative control miRNA (Figure 2D and E).

To further confirm that miR-218 targets BIRC5, direct interaction between miR-218 and the 3′ UTR of BIRC5 mRNA was examined using the luciferase assay. Co-transfection experiments showed that cells transfected with miR-218 had significantly inhibited luciferase activity compared with cells transfected with negative control miRNA (Figure 2F). Moreover, miR-218 did not inhibit the luciferase activity of reporter vector containing the mutant BIRC5 3′UTR in the putative miR-218 binding sites, revealing that miR-218 interacted directly with the 3′ UTR of BIRC5 mRNA.

MiR-218 promotes apoptosis in CRC cells

BIRC5 exerts its effects as an anti-apoptotic gene by means of cell division and chromosome segregation (23). To identify the biological functions of BIRC5 repression by miR-218, we performed
Table 1. Association of miR-218 and clinicopathological factors of CRC patients

<table>
<thead>
<tr>
<th>Gender</th>
<th>High miR-218 expression N (%)</th>
<th>Low miR-218 expression N (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>23 (67.6)</td>
<td>17 (58.6)</td>
<td>0.6</td>
</tr>
<tr>
<td>Female</td>
<td>11 (32.4)</td>
<td>12 (41.4)</td>
<td></td>
</tr>
<tr>
<td>Age, year</td>
<td></td>
<td></td>
<td>0.871</td>
</tr>
<tr>
<td>Median</td>
<td>59</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>34–85</td>
<td>26–79</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td>0.381</td>
</tr>
<tr>
<td>II</td>
<td>7 (20.6)</td>
<td>6 (20.7)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>16 (47.1)</td>
<td>10 (34.5)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>11 (32.3)</td>
<td>13 (44.8)</td>
<td>0.538</td>
</tr>
<tr>
<td>Response (RECIST)</td>
<td></td>
<td></td>
<td>0.0004</td>
</tr>
<tr>
<td>Response</td>
<td>26 (76.5)</td>
<td>9 (31.0)</td>
<td></td>
</tr>
<tr>
<td>Non-response</td>
<td>8 (23.5)</td>
<td>20 (69.0)</td>
<td></td>
</tr>
<tr>
<td>PFS</td>
<td></td>
<td></td>
<td>0.002*</td>
</tr>
<tr>
<td>Median</td>
<td>35</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2.6–40.0</td>
<td>1.3–34.1</td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td>0.0002*</td>
</tr>
<tr>
<td>Median</td>
<td>46</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4.7–55.0</td>
<td>1.5–41.8</td>
<td></td>
</tr>
</tbody>
</table>

N: number; FOLFOX: (5-FU + oxaliplatin + leucovorin); FOLFIRI: (5-FU + leucovorin + irinotecan); 5-FU/LV: (5-FU + leucovorin).
*Statistically significant difference (P < 0.05).

FACS analysis to quantify apoptosis via double staining of cells with annexin V-FITC and PI. The results indicated that miR-218 transfection increased apoptosis significantly compared with cells transfected with negative miRNA in both CRC cell lines tested, while this effect was reversed when BIRC5 was overexpressed with a BIRC5 expression vector in miR-218 transfected cells (Figure 3A and B). To validate that the increase in apoptosis was due to activation of the above-mentioned apoptosis pathway, we determined the nuclear changes and DNA cleavage using the TUNEL assay. Consistent with our expectation, cells transfected with miR-218 showed elevated fluorescence levels of DNA cleavage, and restoration of BIRC5 expression decreased the miR-218-induced DNA cleavage (Figure 3C). These results indicate that miR-218 is a proapoptotic molecule by directly targeting BIRC5.

Overexpression of miR-218 inhibits cell growth of CRC cell lines

To explore the impact of miR-218 on cell proliferation, we compared the proliferation rate of miR-218-transfected CRC cells with that of negative control cells. Cell proliferation was significantly inhibited in miR-218-transfected CRC cells. At day 5, the cell proliferation of miR-218-transfected HT29 and HCT116 cells was reduced by 23 and 55%, respectively (Figure 4A). However, this miR-218-induced proliferation inhibition was reversed by the restoration of BIRC5 expression (Figure 4A). Moreover, the colony formation assay also verified the effect of cell inhibition by miR-218 on CRC cells (Figure 4B). Concomitant with this inhibition of cell proliferation by miR-218, the cell cycle analysis indicated that cell cycle arrest reached significance at the G2 checkpoint in HCT116 cells, and at the S phase checkpoint in HT29 cells (Figure 4C). Thus, miR-218 reduced cell proliferative capacity with G2 or S cell cycle arrest.

miR-218 enhances 5-FU cytotoxicity on CRC cells in vitro

After being transfected with miR-218 precursor or negative control, HT29 and HCT116 cells were incubated without 5-FU or with a concentration gradient of 1, 1.5, 2, 2.5, 3, 4 μM 5-FU for 24, 48 and 72 h. Then, a dose–effect curve was constructed based on the viability of cells treated with or without 5-FU. The graph demonstrated that miR-218 transfection was followed by significantly increased cell death compared with the negative control in 5-FU-treated cells (Figure 5A). To determine whether miR-218 and 5-FU had any synergistic effect, the median inhibitory concentration (IC₅₀) of 5-FU was detected. Our results indicated that the IC₅₀ values for 5-FU were 3.2 and 2.5 μM in non-transfected HT29 and HCT116 cells, respectively. For the cells transfected with miR-218, the IC₅₀ values for 5-FU decreased to 2.2 and 0.9 μM in HT29 and HCT116 cells, respectively (Figure 5B). To sum up, these results suggest that miR-218 has a strong synergistic effect with 5-FU on CRC cell growth.

The anticancer role of 5-FU is activated partially through inhibition of the critical target in cellular proliferation, TS. Thus, we further investigated the correlation between miR-218 and TS protein levels. The results revealed that miR-218 significantly suppressed the expression of TS protein, which explained the potential regulatory mechanism of miR-218 on inhibiting cell proliferation and its synergistic effect with 5-FU on CRC (Figure 5C).

Discussion

Previous studies demonstrated that miR-218 expression was significantly decreased in many malignancies, including glioblastoma (21, 22), nasopharyngeal cancer (24), gastric cancer (25), squamous lung cancer (26) and CRC (19). Consistent with previous studies, our results identified that miR-218 expression was significantly decreased in CRC tissues when compared with the adjacent non-cancerous tissues, and that miR-218 had potential prognostic value in CRC patients treated with 5-FU. More importantly, we identified a novel mechanism of BIRC5 gene suppression mediated by miR-218 in CRC. This suppression of BIRC5 consequently triggered CRC cell apoptosis and inhibition of cell proliferation with cell cycle arrest. It is worth noting that miR-218 also suppresses the expression of the 5-FU target protein TS. As a result, miR-218 triggers apoptosis by suppressing BIRC5 and sensitises CRC cells to 5-FU treatment by suppressing TS protein (Figure 6A).

In our study, we firstly investigated the potential clinical value of miR-218 in the prognosis of 5-FU treatment among CRC patients. Recently, studies have demonstrated the prognostic role of miR-218 in human malignancies, and patients with reduced expression of miR-218 have a higher risk of poor outcome during clinical treatments in gastric cancer, oral cancer, pancreatic ductal adenocarcinoma and CRC (27–29). Our results confirmed the previous findings and further revealed that high miR-218 expression was positively associated with objective response in CRC patients receiving 5-FU-based therapies. In addition, a positive correlation was found between miR-218 expression and OS or PFS, which indicated the prognostic value of miR-218 among CRC patients. This is also of considerable therapeutic significance because a miR-218 restoration method that may provide a new modulation strategy to overcome chemoresistance is important.
Human miR-218 is located in the intronic regions of the SLIT2 gene on chromosome 4p15.2 and SLIT3 on chromosome 5q35.1 (30). Frequent allelic deletion of chromosome 4p15.1–15.3 and 5q35-34 were found in a variety of cancers, including CRC (31). On the other hand, the host genes of the SLIT family are commonly downregulated through promoter hypermethylation in CRC (32). Therefore, the expression of miR-218 was lower in CRC due to allelic loss and hypermethylation. Our study confirmed the expression tendency of miR-218 in CRC and showed that miR-218 promoted apoptosis and inhibited cell proliferation in CRC cells. Furthermore, overexpression of miR-218 can trigger cell cycle arrest in the G2 or S phases. Interestingly, we found that miR-218 expression was inhibited after 5-FU treatment. One potential indicator was that the cells remaining after 5-FU treatment were more resistant to apoptosis, which was consistent with the decreased expression of miR-218. However, this needs further study to obtain a more definite conclusion. Collectively, our results demonstrated that miR-218 might play a role in CRC carcinogenesis as a potential tumour suppressor (Figure 6B). Other studies demonstrated that miR-218 also inhibited cancer cell migration and invasion by targeting laminin-332 in head and neck squamous cell carcinoma (33),

Figure 2. BIRC5 is a direct target of miR-218 in CRC cells. (A) Illustration of the two putative predicted miR-218 binding sites in the BIRC5 3’UTR region. (B) BIRC5 mRNA is decreased following forced expression of miR-218 in CRC tissues. (C) Overexpression of miR-218 was demonstrated in pre-miR-218 transfected CRC cell lines. (D) Western blots showing that miR-218 inhibited BIRC5 protein expression in HCT116 and HT29 cells. (E) BIRC5 mRNA was downregulated in CRC cell lines transfected with miR-218. (F) MiR-218 targets the wild-type but not the mutant 3’UTR of BIRC5 in HCT116 and HT29 cells. Statistically significant difference: * P < 0.05.
induced caspase-mediated apoptosis through interaction with the mammalian target of rapamycin (serine/threonine kinase) (mTOR) rapamycin-insensitive companion RICTOR in oral cancer (34) and suppressed nasopharyngeal cancer progression through downregulation of BIRC5 and the SLIT2-ROBO1 pathway (24), which together proved the clinical significance of miR-218. In a future study, we will examine the effects of demethylating agents (such as azacitidine or decitabine) on 5-FU treatment and miR-218 expression and other functions in CRC cell lines. Moreover, our results determined that miR-218 is a tumour suppressor, mainly acting as a pro-apoptotic miRNA by targeting BIRC5. We demonstrated that BIRC5 is upregulated with the decrease of miR-218 in primary CRC tissues and that it is a direct target of miR-218, with evidence that overexpression of miR-218 led to reduced luciferase activity of the BIRC5 promoter and that miR-218 downregulated BIRC5 mRNA and protein. Moreover, the tumour suppressor role of miR-218 was reversed when the expression of BIRC5 was restored, which proved that the pro-apoptotic effects caused by miR-218 were mediated by directly suppressing BIRC5. It is well known that BIRC5 can inhibit the intrinsic and extrinsic apoptotic pathways by blocking the activity of several caspase proteins. It could be regulated by the ERK and AKT signaling pathways and played a critical role in promotion of tumour mortality and metastasis (35). In addition, BIRC5 protein regulates cell division and forms complexes with chromosomal passenger proteins, including aurora B kinase, INCENP and Borealin (36). Some preclinical researches revealed that the inhibition of BIRC5 was correlated with decreased tumour growth rates in mice (37,38). Several studies also proved that the presence of BIRC5 was strongly associated with the expression of BCL-2 in CRC, gastric cancer (39) and neuroblastoma (40), which means they might function cooperatively during the anti-apoptosis process. More importantly, one major chemoresistance mechanism demonstrated by previous research is the resistance of drug-induced cell death through the attenuation of apoptotic pathways involving BIRC5, thus the blockade of BIRC5 activity has been considered a novel therapeutic strategy to improve chemosensitivity in many cancers (41–44). In short, we validated BIRC5 as a direct target of miR-218 in CRC and the apoptosis process caused by upregulated miR-218 was mediated by the downregulation of BIRC5.

Figure 3. MiR-218 positively regulates CRC cell apoptosis. (A) HT29 and HCT116 cells were transfected with negative control or pre-miR-218 alone, or miR-218 and BIRC5 together, and were stained with annexin V-FITC and PI 48h after transfection. The representative apoptosis pattern was shown and the apoptotic cells were indicated in gate Q4. Analysis of apoptosis shown in Q4 was quantified as in (B). (C) In both CRC cell lines, miR-218 promotes nuclear apoptosis compared with normal control (40×) and BIRC5 restoration reversed this apoptosis. Cells were stained with DAPI and subjected to TUNEL assay to detect DNA and apoptotic cells. Statistically significant difference: *P < 0.01; **P < 0.001.
Resistance to 5-FU treatment is one of the major causes for chemotherapy failure in advanced CRC. Thus, it is essential to better understand the mechanisms of drug resistance and to discover novel strategies to further improve the effectiveness of 5-FU. It is well known that 5-FU suppresses tumour progression mainly by targeting a critical enzyme, TS. TS catalyses the reductive methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, with reduced folate 5,10-methylenetetrahydrofolate as the methyl donor. As this enzymatic reaction, catalysed by TS, is the sole intracellular source of thymidylate, an essential precursor for DNA biosynthesis, the inhibition of TS significantly inhibits cell proliferation. Thus, TS has been a major target of anticancer therapy. Our study showed that miR-218 suppressed the expression of the 5-FU target protein, TS. This indicates another level of synergy to enhance 5-FU chemotherapy by triggering CRC cell apoptosis in addition to knocking down BIRC5. In addition, the IC_{50} for 5-FU in miR-218-transfected CRC cell lines was significantly decreased compared with the normal controls, which further verified the existence of a synergistic effect. Thus, our study might open a new avenue in improving the outcome of chemotherapy. Nevertheless, although we revealed that miR-218 suppressed TS expression, this suppression was not a direct regulation pattern. How miR-218 regulates TS in CRC needs to be studied in further research.

Figure 4. MiR-218 suppresses CRC cell growth. (A) HCT116 and HT29 cells were transfected with either negative miRNA or pre-miR-218 alone or pre-miR-218 and BIRC5 together, and cell numbers were measured with CCK-8 assay. (B) Forced expression of miR-218 led to significantly decreased ability of colony formation in HCT116 and HT29 cell lines. Cell cycle analysis was performed to determine the impact of miR-218 expression. The representative flow cytometry pattern and the G1, S and G2/M ratios were shown in (C). Statistically significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.
Moreover, studies will be done to explore whether forced expression of miR-218 can improve 5-FU chemosensitivity in 5-FU-resistant cell lines in the future.

In conclusion, the present work has identified that miR-218 was related to the response to 5-FU-based treatment in CRC patients and revealed that miR-218 promotes apoptosis by suppressing BIRC5 expression in CRC. MiR-218 also induces inhibition of cell proliferation, causes cell cycle arrest and enhances 5-FU cytotoxicity by suppressing TS protein in vitro. Thus, restoration of miR-218 levels could be a potential novel strategy to enhance chemosensitivity to 5-FU-based treatment.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

Funding

National Natural Science Foundation of China (81301506; 81472025); Research Fund for the Doctoral Program of Higher Education of China (20130131120067); Shandong Province Science and Technology Development Program (2014GSF118016);
References

35. Ye, Q. et al. (2014) ERK and Akt signaling cooperate to transcriptionally regulate survivin expression for metastatic progression of colorectal cancer. Oncogene, 33, 1828–1839.