Original Article

miR-34a increases the sensitivity of colorectal cancer cells to 5-fluorouracil in vitro and in vivo

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Abstract: This study was designed to investigate the significance of the effect of miR-34a on 5-fluorouracil (5-FU) sensitivity in vitro and in vivo. miR-34a expression in tumor tissues or serum was determined by quantitative polymerase chain reaction. CRC cell lines HCT116 and SW480 were used to evaluate cell viability, cell apoptosis, and the cell cycle using a cell proliferation assay, flow cytometry, and Western blotting, respectively. For the in vivo studies, xenografts derived from SW480 cells were established to assess the antitumor activity between miR-34a and 5-FU. Patients with high levels of miR-34a expression were found to benefit more from 5-FU-based chemotherapy than patients with low levels of miR-34a expression, regardless of disease stage. Ectopic expression of miR-34a alone or 5-FU alone was found to inhibit CRC cell growth in vitro and in vivo. Moreover, cell growth in vitro and in vivo was further inhibited when miR-34a combined with 5-FU through increasing the rate of cell apoptosis. The potential targets of miR-34a, including CREB1, Bcl-2, Notch 1, Sirt1, and E2F3, were predicted and preliminarily validated and merit further study. Conclusion: miR-34a might function as a predictor of fluorouracil chemosensitivity in CRC, and a combination strategy of miR-34a with fluorouracil was expected to be more beneficial for CRC patients.

Keywords: miR-34a, colorectal cancer, 5-fluorouracil, chemosensitivity

Introduction

Although the incidence of colorectal cancer (CRC) has gradually increased worldwide, patient prognosis has improved over the past few years [1]. According to CRC guidelines issued by the NCCN, chemotherapy is necessary for patients with stage III or IV CRC and some patients with stage II CRC. Fluorouracil-based chemotherapy has been the most common regimen for CRC over the past fifty years [2, 3]. Allegra et al. reported that the 3-year disease-free survival rates (3-year DFS) of stage II and III CRC patients who received fluorouracil-based adjuvant chemotherapy were 85.4% and 71.7%, respectively [4]. Among stage IV CRC patients, about 55.0% responded to systematic chemotherapy [5]. These data suggested that there was readily visible heterogeneity between patients [4-8], and that predictive markers were needed to guide adjuvant or systematic chemotherapy, followed by optimizing strategies to enhance sensitivity to chemotherapy.

microRNAs (miRNAs) constitute a class of non-coding RNA molecules of approximately 18-25 nucleotides in length [9, 10]. Increasing amounts of evidence have indicated that microRNAs (miRNAs) play various roles in multiple cancers, including CRC [9-13]. miRNAs can be easily detected from a variety of samples, such as tissues, plasma, serum, and stools [14]. Our previous study demonstrated that miR-34a was downregulated in CRC tumor tissues, and miR-34a expression was positively correlated with disease-free survival (DFS) in stage II/III CRC patients [15]. In our study we found that patients with low levels of miR-34a tend to early recurrence than patients with high levels of miR-34a among patients who received fluorouracil-based adjuvant chemotherapy (detailed results are shown in the text). It has been suggested that miR-34a has some synergistic effect on fluorouracil.
Akao Y et al reported similar findings, specifically that miR-34a dysregulation could induce 5-fluorouracil (5-FU) resistance in human CRC DLD-1 cells [16]. This study was designed to establish the association between miR-34a and 5-FU through analysis of the relationship of miR-34a expression to clinical response in patients treated with fluorouracil-based chemotherapy. We conducted an *in vitro* cell experiment and *in vivo* animal experiment to assess the influence of miR-34a on the sensitivity to 5-FU in CRC and to determine its underlying mechanism.

**Materials and methods**

*Patients and sample collection*

Data concerning patients with stage II/III cases were extracted directly from our previous study [15]. Here, 37 CRC patients with stage IV treated in our department from July 2013 to June 2015 were included in this study. These 37 patients provided serum samples prior to chemotherapy. All serum samples were stored at -80°C for future research. The patients received first-line fluorouracil-based chemotherapy. The clinical data mainly included patients’ clinical responses, which were obtained from their medical records. Clinical response evaluation was performed by computed tomography (CT) scan according to the RECIST 1.1 criteria. Response evaluation included partial response (PR; at least a 30% decrease in the sum of diameters of target lesion), progressive disease (PD; at least a 20% increase in the sum of diameters of target lesions), and stable disease (SD; neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD). All patients provided written informed consent for their samples to be used in research, and this study was approved by the ethics committee of Peking University Cancer Hospital.

*Cell lines and antibodies*

Seven human CRC cell lines were used here: Caco 2, HT29, HCT116, HCT116 p53-/-, Lovo, SW620, and SW480. Caco 2, HT29, HCT116, Lovo, SW620, and SW480 were purchased from ATCC and cultured in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, U.S.) supplemented with 10% fetal bovine serum (Gibco BRL). HCT116 p53-/- was provided by Professor Bert Vogelstein of Johns Hopkins University and cultured in McCoy’s 5A modified medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL). All cell lines were incubated in a humidified 37°C incubator under 5% CO₂. Antibodies of cleaved caspase 9 (#7237), cleaved caspase 8 (#8592), cleaved PARP (9532), and Bcl-2 (#15071) were purchased from Cell Signal Technology (CST, Danvers, MA). Antibody of β-actin (#014M4759) was purchased from Sigma-Aldrich (St. Louis, MO).

*Analyses of miR-34a expression*

Data of miR-34a expression from tumor tissues was collected from our previous reported study. RNA extraction from serum samples and cell line was conducted using a Trizol LS kit (Invitrogen, Carlsbad, CA, U.S.) and Trizol (Invitrogen) according to the manufacturers’ instructions. After normalization with endogenous control RNU6B, miR-34a expression was evaluated, which was described in previous reports [15].

*Cell viability assay*

HCT116 and SW480 cells were transfected with miR-34a or negative control miR-Ctrl (GenePharma, Shanghai, China) using lipofectamine 2000 (Invitrogen). After 48 h of transfection, cell viability was analyzed using a Cell Counting Kit-8 (CCK8, Dojindo) according to the manufacturer’s instructions. Absorbance was measured at 450 nm using the microplate spectrophotometer once a day for 5 consecutive days. All reactions were run in triplicate.

*Annexin V apoptosis assay*

HCT116 and SW480 cells transfected with miR-34a or negative control miR-Ctrl were harvested and stained with phycoerythrin (PE)- annexin V and 7-amino-actinomycin (7-AAD) (BD Biosciences, Erembodegem, Belgium) at room temperature in the dark for 15 min. Cells were then detected using flow cytometry and analyzed using FlowJo 7.6 software (FlowJo, LLC.).

*Cell cycle assay*

Forty-eight hours after transfection with miR-34a or miR-Ctrl, cells were harvested and fixed in 70% cold ethanol for at least 12 h at 4°C. Fixed cells were treated with DNase-free RNaseA at 37°C for 30 min followed by staining with 50 μg/mL propidium iodide (BD Biosci-
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ences) at room temperature in the dark for 30 minute. Cells were then detected by FACS Calibur system (BD Biosciences) and results were analyzed with ModFit 3.0 software (BD Biosciences).

**Western blot analysis**

Total protein was extracted from cell or tissue samples using RIPA Lysis Buffer (Beyotime, Shanghai, China) on ice. After the determination of protein concentration, about 50 micrograms of protein was separated on 10% SDS-PAGE, after which protein was transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, U.S.). Nitrocellulose membrane was then incubated with primary antibodies overnight at 4°C and secondary antibody at room temperature for 1 h (antibodies are depicted in Supplementary Table 1). Proteins were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare).

**Prediction and validation of targets of miR-34a**

In this study, three online target prediction software packages including TargetScan (http://www.targetscan.org/), DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/)

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients in cohort I</th>
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<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
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<td>Age (years)</td>
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<td>Differentiation*</td>
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<td>Depth of penetration</td>
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<tr>
<td>N1 (n = 28)</td>
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<td>Lymph-vascular Invasion</td>
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<tr>
<td>Yes (n = 21)</td>
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<td>No (n = 70)</td>
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</tbody>
</table>

*Good including well-differentiated and moderately differentiated adenocarcinoma; Poor including poor-differentiated adenocarcinoma, mucinous adenocarcinoma, and signet ring cell carcinoma.

**Tumor experiments**

SW480 cells with miR-34a and miR-Ctrl expression were established using a lentivirus system (GenePharma, Shanghai, China) and injected subcutaneously into the dorsal flanks of 6-week-old NOD/SCID mice (Beijing HFK Bio-Technology Co, LTD., Beijing, China) to establish SW480-derived xenografts. When tumors reached approximately 150 mm³, mice were randomly divided into two groups (n = 5 per group): 5-FU group (15 mg/kg, intraperitoneal injection, twice a week for 2 weeks) and control group (0.9% saline solution, intraperitoneal injection, twice a week for 2 weeks) for xenografts with miR-34a or miR-Ctrl expressions. Tumor size and body weight were measured with digital calipers and electronic scale every three days, and the tumor volume was calculated using the following formula: Volume = 0.5 × (Length × Width²), where length was the long diameter and width was the short diameter of xenografts. The antitumor activity was depicted by tumor growth inhibition (TGI). TGI was calculated as the following formula: TGI = ΔT/ΔC × 100% (ΔT = tumor volume change of experimental group indicated by tumor volume at the final day of study minus tumor volume at the initial treatment, ΔC = tumor volume change of control group indicated by tumor volume at the final day of study minus tumor volume at the initial treatment). Animal experiments were approved by the independent ethics committee at Peking University Cancer Hospital.
miR-34a increases sensitivity of CRC to 5-FU

The differences in miR-34a expression between different groups were analyzed using Wilcoxon rank-sum tests. Wilcoxon rank-sum tests were also used to analyze the correlations of miR-34a expression with cell viability, cell apoptosis, and tumor growth. A two-side \( P \) value <0.05 was considered statistically significant.

Results

miR-34a expression acts as a predictor for fluorouracil sensitivity in CRC patients

The correlation between miR-34a expression in tumor tissues and 3-year recurrence rate was analyzed in cohort 1 including 91 patients with stage II/III CRC. The characteristics of patients including gender, age, primary tumor sites, differentiation, depth of penetration, and lymph node metastasis were listed in Table 1. According to our previous study, we set the expression level of 0.307-2866 as a cutoff value [15], and miR-34a expression higher than the cut-off level was defined as high expression. Among patients with adjuvant chemotherapy, the 3-year recurrence rate in patients with high levels of miR-34a expression was significantly lower than in patients with low levels of miR-34a expression (37.0\% vs. 75.0\%, \( P \)<0.01; Figure 1A). These results suggested that miR-34a expression was closely related to the patient response to fluorouracil-based adjuvant chemotherapy.

To further validate the hypothesis given above, we analyzed the association between miR-34a expression and clinical response in 37 stage IV CRC patients treated with first-line fluorouracil-based regimens. The basic characteristics of patients with stage IV CRC and clinical response evaluations were shown in Table 2. The num-

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**Table 2. Characteristics of patients in cohort II**

<table>
<thead>
<tr>
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<th>Partial response (n = 13)</th>
<th>Disease stable (n = 19)</th>
<th>Progressive disease (n = 5)</th>
</tr>
</thead>
<tbody>
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<td><strong>Age, years</strong></td>
<td>Median (range)</td>
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<td></td>
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<tr>
<td>Female/Male</td>
<td>7/6</td>
<td>9/10</td>
<td>3/2</td>
</tr>
<tr>
<td>Tumor site</td>
<td>Colon/Rectum</td>
<td>11/2</td>
<td>14/5</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Good/Poor</td>
<td>11/2</td>
<td>16/3</td>
</tr>
</tbody>
</table>

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The correlation between miR-34a expression in tumor tissues and 3-year recurrence rate was analyzed in cohort 1 including 91 patients with stage II/III CRC. The characteristics of patients including gender, age, primary tumor sites, differentiation, depth of penetration, and lymph node metastasis were listed in Table 1. According to our previous study, we set the expression level of 0.307-2866 as a cutoff value [15], and miR-34a expression higher than the cut-off level was defined as high expression. Among patients with adjuvant chemotherapy, the 3-year recurrence rate in patients with high levels of miR-34a expression was significantly lower than in patients with low levels of miR-34a expression (37.0\% vs. 75.0\%, \( P \)<0.01; Figure 1A). These results suggested that miR-34a expression was closely related to the patient response to fluorouracil-based adjuvant chemotherapy.
miR-34a increases sensitivity of CRC to 5-FU

The expression levels of miR-34a were measured in CRC cells and compared to normal colon tissues. The results showed higher expression of miR-34a in CRC cells (Figure 2A). Ectopic expression of miR-34a enhanced the ability of cell growth inhibition of 5-fluorouracil (5-FU) (Figure 2B). The combination of miR-34a and 5-FU resulted in significantly lower viability in CRC cells (Figure 2C and 2D).

The response of patients to 5-FU treatment was classified as partial response (PR), stable disease (SD), or progressive disease (PD). The distribution of patients was as follows: 13 (35.1%) for PR, 19 (51.4%) for SD, and 5 (13.5%) for PD. Patients with PR had higher levels of miR-34a expression compared to those with SD or PD. Patients with high levels of miR-34a expression could benefit more from fluorouracil-based chemotherapy than other patients. This suggested that miR-34a expression could predict patient response to fluorouracil and that there could be some synergy between miR-34a and fluorouracil.

Ectopic expression of miR-34a enhanced the ability of cell growth inhibition of 5-fluorouracil

There was less miR-34a expression in CRC tissues than in matched non-tumor tissues in our previous study, which we here confirmed.
miR-34a increases sensitivity of CRC to 5-FU
miR-34a increases sensitivity of CRC to 5-FU

Compared to three normal colon tissues that served as surrogates due to the absence of normal colon cell lines, miR-34a expression was significantly downregulated in seven CRC cell lines (Figure 2A). As shown in a previous study, both miR-34a and 5-FU alone could inhibit the growth of CRC cells. The current work addresses whether miR-34a has any synergistic effect with 5-FU. HCT116 and SW480 cell lines were used in the following experiments, and ectopic expression of miR-34a was confirmed by quantitative polymerase chain reaction (PCR; Figure 2B). After treatment, cell viability indicated by OD$_{450}$ was highest in cells treated with miR-Ctrl (the mean OD$_{450}$ was 6.49 in HCT116 cell and 10.47 in SW480 cell), moderate in cells treated with miR-34a (the mean OD$_{450}$ was 3.55 in HCT116 cell and 6.20 in SW480 cell), and lowest in cells treated with miR-34a+5-FU (the mean OD$_{450}$ was 0.98 in HCT116 cell and 2.42 in SW480 cell). These results suggested that miR-34a could increase the sensitivity of CRC cells to 5-FU in vitro (Figure 2C and 2D).

miR-34a increased 5-FU sensitivity by inducing cell apoptosis

To explore the mechanisms by which miR-34a increased 5-FU sensitivity, we used flow cytometry to analyze the cellular apoptotic rate. As shown in Figure 3A-D, compared to control...
miR-34a increases sensitivity of CRC to 5-FU

(8.1% and 13.7% in HCT116 and SW480), miR-34a (16.8% and 17.5% in HCT116 and SW480) or 5-FU (10.5% and 16.1% in HCT116 and SW480) alone could increase the proportion of apoptotic cells in both cells. Moreover, more apoptotic cells in both cells were found when miR-34a combined with 5-FU (20.5% and 19.8% in HCT116 and SW480). Induction of cell apoptosis was further confirmed by western blot to detect apoptosis-related proteins. Accompanied by cell apoptosis, the expression of cleaved caspase-9, caspase-8 and PARP was enhanced, but the anti-apoptosis gene Bcl-2 was inhibited (Figure 3E). These data suggested that induction of cell apoptosis was one of the mechanisms involved in miR-34a expression increasing 5-FU sensitivity.

miR-34a increased the inhibitory activity of 5-FU in vivo

SW480 cells transfected with miR-34a or miR-Ctrl were confirmed by quantitative PCR (Figure 4A) and then subcutaneously inoculated into the dorsal flank of NOD/SCID mice to establish xenografts in vivo. After the tumors reached about 150 mm³, mice were treated with 5-FU or 0.9% saline solution as a control. We found that, unlike in cells transfected with miR-Ctrl and treated with 0.9% saline solution, the growth of tumors in other three groups was suppressed to a certain extent. Tumor suppression was most significant in cells transfected with miR-34a and treated with 5-FU, with tumor growth inhibition (TGI) 91% (Figure 4B and 4C), which was consistent with the results shown in Figure 2 and confirmed that miR-34a increased the antitumor activity of 5-FU in CRC in vitro and in vivo.

As presented above, cell apoptosis was also analyzed using Western blot analysis to detect the expressions of apoptosis-related proteins in xenografts. As shown in Figure 4D, cell apoptosis was confirmed according to the changes in cleaved caspase-9, caspase-8, and Bcl-2.
Potential target genes of miR-34a

To facilitate further study of the underlying mechanism, we predicted possible targets of miR-34a by searching three reliable databases (MicroRNA-org, DIANA, TargetScan). Figure 5A lists several target genes including CREB1, Bcl-2, Notch 1, Sirt1, E2F3, HDAC1, and BIRC5, which were reported to be involved in the regulation of 5-FU chemosensitivity [15-20], and the binding sites of miR-34a in 3'-UTR were also presented in Figure 5B. Our results demonstrated that the expression of CREB1, Bcl-2, Notch 1, Sirt1, and E2F3 was decreased in HCT116 cells transfected with miR-34a compared to miR-Ctrl (Figure 5C), which suggested these genes might be the targets of miR-34a. However, whether these genes are the direct targets of miR-34a remains unclear and needs to be validated further.

Discussion

miR-34a has been confirmed to be downregulated in multiple tumors, including CRC, in several studies [15, 21-24]. We previously demonstrated that the expression of miR-34a can serve as an independent prognostic factor for recurrence [15]. In this study, we analyzed the 3-year recurrence rate of 91 stage II/III CRC patients who received fluorouracil-based adjuvant chemotherapy stratified by miR-34a expression. We found that patients with high levels of miR-34a had lower 3-year recurrence rate than patients with low levels of miR-34a. It is here suggested that miR-34a has some relationship to fluorouracil.

To validate the above, we evaluated patients with stage IV CRC treated with first-line fluorouracil-based regimens. As expected, patients with high levels of miR-34a expression were found to benefit more from fluorouracil-based chemotherapy than patients with lower levels of expression. Our results indicate for the first time that miR-34a expression could predict the patient response to fluorouracil-based chemotherapy in patients. In clinical practice, almost all patients received fluorouracil as part of their combination regimens. As a consequence, our results suggested that there might be some correlation between miR-34a and fluorouracil, which we confirmed using in vitro cell experiments and in vivo animal experiments.

Our cell viability assay in HCT116 and SW480 cells demonstrated that cell growth was significantly inhibited by miR-34a combined with 5-FU relative to miR-34a or 5-FU alone. This was further confirmed in SW480-cell-derived xenografts. These results suggested that miR-34a could increase 5-FU therapeutic sensitivity in vitro and in vivo. Consistent with our results, other groups reported that miR-34a was downregulated in 5-FU-resistant DLD-1 cells, and that miR-34a could resensitize 5-FU-resistant DLD-1 cells to 5-FU. All of this data showed that miR-34a was promising for use in patients to enhance the sensitivity of 5-FU treatments.

To identify the mechanisms involved in the increased effects of miR-34a and 5-FU, we evaluated two important processes: the cell cycle and cell apoptosis. Consistent with our previous study, significant induction of miR-34a-mediated apoptosis via the caspase apoptosis pathway was verified in this study. There was less overall 5-FU-mediated apoptosis than miR-34a apoptosis, but the most apoptotic cells were observed when miR-34a and 5-FU were administered in combination than miR-34a or 5-FU alone in both cell lines. There were also differences in the make-up of apoptosis-related proteins, which were assessed in both in vitro cells (Figure 3) and in vivo xenografts (Figure 4).

In regard to cell cycle, compared to control, miR-34a was confirmed to induce cell cycle arrest at G1 phase, however, 5-FU was verified to induce cell cycle arrest at S phase. As a result, the progress of the cell cycle in cells exposed to both miR-34a and 5-FU might be complicated and the results may be difficult to interpret, which was shown in Supplementary Figure 1.

MicroRNA acts by regulating target genes, and several target genes have been reported to be regulated by miR-34a, such as Notch1, c-Myc, Sirt1, Bcl-2, LDHA, and KLF4. In this study, three reliable databases (MicroRNA-org, DIANA, TargetScan) were employed to predict the possible target genes of miR-34a. Seven potential targets including CREB1, Bcl-2, Notch 1, Sirt1, E2F3, HDAC1, and BIRC5 were screened and preliminarily validated. We found that ectopic expression of miR-34a could not suppress the expression of HDAC1 or BIRC5 at the mRNA
miR-34a increases sensitivity of CRC to 5-FU

level. Among the remaining five genes downregulated by miR-34a, further studies would be conducted to confirm the genes directly regulated by miR-34a and involved in 5-FU sensitivity. It was reported that Bcl-2 was an integral outer mitochondrial membrane protein that blocked the apoptotic death of some cells such as lymphocytes [27]. Moreover, numerous studies reported that Bcl-2 participated in regulating chemosensitivity in multiple tumors [17]. Wu and his colleagues reported that the phosphorylated Bcl-2 regulated by PXN contributed to 5-FU based chemotherapy resistance in CRC [17]. Similarly, numerous studies indicated that CREB1, Notch 1, Sirt1, and E2F3 were reported to play a role of oncogenes and involved in regulating response to chemotherapy [16, 18-22]. Further studies would be conducted to identify the direct genes regulated by miR-34a and involved in 5-FU sensitivity.

Also, there are limitations in our study and the results were needed to be further validated due to the relative small samples of stage IV CRC patients. In addition, more mechanisms underlying cell proliferation and chemosensitivity would be investigated such as autophagy and necrosis, and the identification of target genes of miR-34a would be continued. In summary, we found for the first time that miR-34a might function as a predictor of fluorouracil chemosensitivity in patients with CRC, and a combination strategy of miR-34a with fluorouracil was expected to be more beneficial for CRC patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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**Supplementary Table 1.** Primer sequences and size of targets of miR-34a

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<th>Reverse</th>
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<td>Bcl-2</td>
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<td>E2F3</td>
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Supplementary Figure 1. Cell cycle analysis in HCT116 and SW480 cells treated with miR-Ctrl, miR-34a, miR-Ctrl+5-FU, and miR-34a+5-FU.