miR-20a/Foxj2 Axis Mediates Growth and Metastasis of Colorectal Cancer Cells as Identified by Integrated Analysis

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Background:
MicroRNAs (miRNAs) have a significant regulatory effect on the proliferation, migration, and invasion of cells, and have been widely reported to have oncogenic or tumor-suppressive impacts on various tumors. In the present study, we assessed the regulation and function of miR-20a on colorectal cancer (CRC) cell lines.

Material/Methods:
qPCR was used to quantify miR-20a expression. Luciferase reporter assay was conducted to confirm Foxj2 3’UTR associations. In addition, the function of miR-20a and Foxj2 in CRC was detected using MTT, colony formation, transwell assays, and cell cycle analysis.

Results:
Our data revealed that miR-20a expression was elevated in the CRC cell lines, and cell migration, proliferation, and invasion abilities were promoted by the overexpression of miR-20a. Moreover, Foxj2 was authenticated as a direct target gene of miR-20a in CRC cells. Furthermore, we found that the ectopic Foxj2 dramatically suppressed miR-20a-promoted proliferation, migration, invasion, and xenografts in vitro and in vivo, and induced cell cycle arrest at G1 stage.

Conclusions:
Our results showing the roles of miR-20a/Foxj2 in carcinogenesis of CRC may help improve treatment of CRC.

MeSH Keywords:
Colorectal Neoplasms, Hereditary Nonpolyposis • MicroRNAs • Tumor Markers, Biological

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Colorectal cancer (CRC) is a common malignant tumor that is one of the leading causes of cancer-related death worldwide [1]. In developed Western countries, CRC is the second most common cancer, and morbidity and mortality rates are continually increasing in China [2]. The onset of CRC is subtle, early symptoms are mild or inconspicuous; therefore, many patients are diagnosed in the middle and late stages of CRC, with poor response to treatment unfavorable prognosis. The 5-year survival rate of advanced metastatic CRC is less than 10%, and it has the fifth-highest cancer mortality rate in China. Although increasing understanding, improved diagnosis, and more effective therapy of CRC, the precise molecular mechanisms underlying disease initiation and progression are complicated and ambiguous. Therefore, it is of great clinical significance to identify biomarkers related to CRC.

miRNAs repress gene expression, mainly by direct interaction with the 3'-untranslated regions (3'UTRs) of mRNAs, and belong to a cluster of highly conserved, small, non-coding RNAs [3]. miRNAs can serve as either tumor oncogenes or inhibitors, and take part in many biological processes, including cell proliferation, differentiation, and apoptosis, by targeting various transcripts, and are associated with the initiation, promotion, and development of all types of human cancer [4]. With the development of molecular genetics, growing evidence proved that miR-20a is expressed at higher levels in CRC tissues than in normal adjacent mucosal tissues, and patients with elevated expression levels of miR-20a have larger lymph nodes. The high incidence of distant metastasis suggests that miR-20a can regulate the metastasis of CRC. Li et al. indicated that the level of miR-20a was considerably higher in CRC tissues and is correlated with the apoptosis and distant metastasis of cancer cells. Additionally, miR-20a plays a role in migration, invasion, and epithelial-to-mesenchymal transition (EMT) of CRC cells by regulating the underexpression E-cadherin and overexpression of vimentin. However, the mechanism through which miR-20a affects the development of CRC is unclear.

In the present work, integrated analyses were performed and miR-20a was screened as an oncogenic molecule in CRC, suggesting that the level of miR-20a was upregulated in CRC cells and tissues. The overexpressed miR-20a positively regulated cell proliferation and colony formation. Additionally, Foxj2 was identified as a hub target of miR-20a based on the integrated bioinformatics analysis and in vitro experiments. Subsequently, the regulation of miR-20a on Foxj2 was also verified, showing that cell migration and invasion were promoted by miR-20a by suppressing the expression of Foxj2.

### Material and Methods

#### Integrated analysis of CRC miRNA and mRNA datasets

The gene and miRNA expression data and the relevant clinical information of TCGA-COAD were extracted from The Cancer Genome Atlas (TCGA) database in April 2019. The EdgeR package was used to estimate differentially expressed miRNA between CRC tissues and normal tissues. \(|\text{logFC}| > 1\) and \(FDR < 0.001\) were deemed to be statistically significant. We also identified the cluster of differently expressed miRNAs and mRNAs based on the “pheatmap” package in R.

#### Prediction of miR-20a targets and enrichment analysis

The potential targets of miR-20a were identified based on the intersection of 3 online databases: miRTarBase [5], miRDB [6], and TargetScan [6]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using the clusterProfiler package [7]. The correlation between miR-20a and Foxj2 was assessed and plotted using the online starBase [8].

#### Tissue specimens and cell culture

Paired CRC tissues and its tumor-adjacent tissues were acquired from patients undergoing radical resection at the First People’s Hospital of Jingmen from 2015 to 2016. We randomly selected 24 patients, and the study was approved by the Ethics Committee of the First People’s Hospital of Jingmen.

The human CRC cell lines SW480, SW620, HCT-116, and HCT-8, as well as the normal intestinal epithelial cell line FHC, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco) containing 10% fetal bovine serum (FBS, Gibco) was used to culture the SW480 and HCT-8 cells in a humidified atmosphere with 5% CO\(_2\) at 37°C. SW620 and HCT-116 cells were incubated in DMEM (Gibco) containing 10% FBS (Gibco) with the same conditions.

#### Western blot analysis

Total proteins were separated from the CRC tissues and cells. The concentrations of proteins were identified based on the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the proteins, which were then transferred to PVDF membranes (Millipore). Then, the membrane proteins were subjected to immunoblotting with appropriate antibodies following the instructions of the manufacturer. The protein bands were visualized and quantified.
Real-time quantitative PCR
TRIZOL reagent (Invitrogen) was used to isolate total RNA from frozen tumor tissues and cells. We used the cDNA Synthesis Kit (Takara, Tokyo, Japan) for synthesis of cDNA. The qPCR for miRNAs and mRNA was conducted with specific primers following the instructions of the manufacturer (Life Technologies, Carlsbad, CA, USA). QPCR results of miRNA and mRNA were expressed corresponding to U6 snRNA or β-actin mRNA, respectively. The sequences of the primers were: Foxj2: 5’-TATGGTAGGCATAGGACAA-3’ (forward), 5’-GCAAATTAAGAGGGAGACA-3’ (reverse); β-actin: 5’-TAGTGGTTACACCCCTTCTTG-3’ (forward), 5’-GCTGTCACCTTCAGCTCC-3’ (reverse); miR-20a: 5’-ACACTCCAGCTGGGTAAAGTGCTTATAGTGCA-3’ (forward), 5’-GCTGTCACCTTCAGCTCC-3’ (reverse); U6: 5’-CTCGCTTCGGCAGCACA-3’ (forward), 5’-AAGCTTCCAGAATTTGC-3’ (reverse).

Establishment and transfection of plasmid
The overexpression of miR-20a was performed by transfecting a miR-20a mimic with a synthesized double-stranded RNA oligonucleotide imitating the miR-20a precursor. The synthesized miR-20a mimic and the random negative control RNAs (control mimic and inhibitor) were obtained from GenePharma (Shanghai, China). For overexpression of endogenous Foxj2, the coding sequence of Foxj2 was amplified and subcloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The empty plasmid was used as the negative control (plasmid control). The overexpressed plasmids were transfected into the cells by Lipofectamine 2000 (Invitrogen) based on the protocols.

Luciferase reporter assay
HCT-116 cells were seeded in 24-well plates with a density of 1×10⁴ cells/well, which were further incubated for 24 h prior to transfection. When performing the reporter gene experiments, 0.5 μg of pGL3-Foxj2-3’UTR or pGL3-Foxj2-3’UTR Mut plasmid, 0.05 ng of the pRL-TK control vector (Promega, USA), and 100 nM miR-20a or the control RNA were used to collectively transfect the cells based on Lipofectamine 2000 (Invitrogen, USA). The Dual-Luciferase Reporter Assay kit (Promega) was used to explore the luciferase activities based on the instructions of the manufacturer. The normalization was assessed by the Renilla luciferase activity.

Transwell assay
Cell migratory and invasive capacities were determined by transwell chamber assay (BD Biosciences). The transfected cells were collected 24 h after transfection. Subsequently, 3.0×10⁴ transfected cells or control cells were added to every upper insert in the serum-free medium. We added 500 μL culture medium supplemented with 10% FBS to the lower chamber. After the cultivation, non-migrated and non-invaded cells were discarded from the top surface of the transwell membrane using a cotton swab, and the cells remaining on the substrate were fixed with methanol, stained with 0.1% crystal violet, and photographed and counted. The above experiments were conducted in triplicate with biological samples.

Cell cycle analysis
Cell cycle analysis was conducted after 48 h of the miRNA transfection based on flow cytometry. Briefly, 2×10⁶ cells were harvested and trypsinized, re-suspended in ethanol, and stored at 4°C for 15 min. Afterward, cells were centrifuged and re-suspended in 450 μL PBS and 50 μL RNase A at 37°C for 30 min. Cells were then treated with propidium iodide (PI, 50 μg/mL) and were cultured for 30 min in the dark at room temperature. Cell cycle analysis was achieved on a FACScan flow cytometer (BD Biosciences) using ModFit LT software.

Cell apoptosis
The Annexin V-FITC/PI staining kit (BD Biosciences, San Diego, CA, USA) was used for cell apoptosis. HCT-116 cells were seeded in 6-well plates with a density of 10⁵ cells/mL. After transfecting for 24 h, Annexin V-FITC was used to label the cells for 20 min in the darkness. In total, 50 μg/mL of PI was added and further incubated for 30 min. Cell apoptosis was detected to calculate the percentage of cell death based on flow cytometry with the FACScan flow cytometer (BD Biosciences). All tests were performed in triplicate.

Cell proliferation and colony formation assay
For cell proliferation, cell viability was assessed by the MTT assay, in which 3.0×10⁴ cells per well were seeded in 24-well plates and cultured in complete medium. Subsequently, cells were treated with 20 μL MT5 (5 mg/mL) and cultured for 4 h. After discarding the medium, the formazan crystals were re-suspended in dimethylsulfoxide (DMSO) and the absorbance was immediately assessed using a microplate reader at 490 nm. For the colony formation assay, 1×10⁴ cells were seeded in 6-well culture plates and cultured in complete culture medium. After culturing for 15 days, 3.7% methanol was applied to fix the colonies of living cells, 0.1% crystal violet was applied for staining, and cell colonies were counted. Colonies consisting of at least 50 cells were scored. All the above experiments were conducted in triplicate.
Xenograft study

For in vivo analyses, cells (1×10⁶) were suspended in 100 μL serum-free DMEM and subcutaneously injected into the flanks of BALB/c male nude mice (3–4 weeks old). For each group, 5 mice were included. The development of tumors in mice was observed every 3 days. Four weeks later, the mice were euthanized and the tumors were removed and photographed, and the volumes were calculated based on the following formula: length (mm)×width² (mm²)/2.

Statistical analysis

Univariate Cox proportional hazards regression analysis was performed for the identification of candidate genes closely associated with survival. Of the screened genes, the candidates with p-value <0.001 were further subjected to multivariate Cox proportional hazards regression analysis to select the prognostic gene markers. SPSS 17.0 software (SPSS, Chicago, IL) was used for all the statistical analyses. The results are expressed as mean±standard deviation with no less than 3 biological repeats. The comparison between the 2 groups was performed by t test. One-way ANOVA was used to assess the significance of variations in different groups. P-values <0.05 were deemed to be statistically significant.

Results

miR-20a was overexpressed in CRC and upregulated cell proliferation and colony formation

The statistical software R and EdgeR packages were used to identify the differentially expressed microRNAs between control and tumor tissues. Figure 1A displays the volcano plot. All of the altered miRNAs expressed in CRC tissues were identified (Figure 1B), showing that the expression of miR-20a was higher in CRC. RT-qPCR analysis showed that the levels of miR-20a were elevated in 4 CRC cell lines – SW480, SW620, HCT-8, and HCT-116 – compared with the normal cell line FHC (Figure 1C). Additionally, it was revealed in Figure 1D that the level of miR-20a expressed in CRC tissues was higher in CRC tissues than in the relevant para-carcinoma tissues.

To further explore the roles of miR-20a in the development of CRC, MTT and colony formation assays were performed in vitro. miR-20a was overexpressed in HCT-116 cells transfected with miR-20a mimics (Figure 1E). MTT assay showed that cell proliferation was facilitated by overexpression of miR-20a (Figure 1F). Colony formation assay showed that overexpression of miR-20a facilitated the colony-forming ability of HCT-116 cells (Figure 1G). The number of colonies developed from cells treated with miR-20a mimics was counted, which was dramatically higher than that of scramble or control cells (* P<0.05).

Identification of miR-20a candidate target genes

To assess the abnormal regulation of miR-20a on CRC, the potential mRNA targets of miR-20a were obtained by the verified target module of miRDDB, miRTarBase, and TargetScan databases. Eventually, 120 candidates were selected from these 3 databases. Univariate Cox regression analysis and stepwise multivariate Cox regression analysis were performed to assess the relationship between differentially expressed genes and the overall survival of patients with CRC. As represented in Supplementary Table 1, 228 downregulated genes with p-value <0.001 were considered as the prognostic candidates, which were chosen for the next screening. To authenticate the reliable target genes, the intersection of promising miR-20a target genes and 228 reduced differentially expressed genes were extracted, which eventually screened out 3 target genes (FOXJ2, CRY2, and PLS1), among which, FOXJ2 has never been previously studied in CRC (Figure 2A).

We assessed the correlation between miR-20a and the candidates using the combination of GO analysis and KEGG pathway analysis. GO enrichment results (Figure 2B) suggested that the activation of MAPKK was the most enriched pathway. The KEGG results (Figure 2C) suggested that the candidates are generally involved in development of a variety of cancers and EGFR tyrosine kinase inhibitor resistance. Moreover, we also developed the expression heatmap for all potential target genes of miR-20a (Figure 2D). The GEPIA database also verified that the FOXJ2 expression was inhibited in tumors in comparison with normal tissues (Figure 2E). The results of starBase (Figure 2F) suggested that the level of FOXJ2 was negatively correlated with that of miR-20a.

FOXJ2 serves as the direct target of miR-20a in CRC cells

To sequentially identify the putative target gene of miR-20a in CRC, FOXJ2 was utilized as a possible target gene of miR-20a (Figure 3A). To further confirm FOXJ2 as a target of miR-20a, the miR-20a binding site was speculated to be in the 3'UTR of FOXJ2 mRNA, which was cloned into the dual-luciferase reporter vector. Subsequently, either miR-20a or the negative control RNA with the dual-luciferase expression vector including wild-type or mutant 3'UTR fragment of FOXJ2 was co-transfected. Dual-luciferase reporter assay was performed to validate that the luciferase activity of the wild-type FOXJ2 3'UTR luciferase complex rather than the mutant FOXJ2 3'UTR was dramatically suppressed by the overexpressed miR-20a (Figure 3B) (P<0.05). The above results show that FOXJ2 directly interacts with miR-20a. In addition, miR-20a was transfected to the HCT-116 cells, and the results of Western blot analyses proved...
Figure 1. miR-20a expression was upregulated in CRC and overexpression of miR-20a promoted cell growth and colony formation. (A) Volcano plot of differentially expressed miRNAs. X-axis: log₂ (fold change); Y-axis: log₁₀ (FDR). Red dots: significantly upregulated genes; Green dots: significantly downregulated genes; Black dots: non-differentially expressed genes. (B) The heatmap of the differentially expressed miRNAs between CRC tissues and adjacent normal tissues. Differentially expressed genes in CRC were screened and are presented in the rows, and the columns are samples. The green represents downregulation, while a red represents upregulation. (C) The expression of miR-20a was detected in CRC cell lines (SW480, SW620, HCT-8, and HCT-116) and the normal cell line FHC using qPCR analysis. (D) qPCR analysis of miR-20a expression levels in 24 pairs of CRC tissues and their corresponding adjacent normal tissues (P<0.001). (E) qPCR analysis was used to evaluate miR-20a expression after transfection with miR-20a mimics or scramble or no transfection (control). (F) MTT assay was used to study the proliferation of the HCT-116 cells after transfection with the miR-20a mimics or scramble or control. (G) Overexpression of miR-20a promoted the HCT-116 cells colony formation. The relative numbers of colonies are shown in the right. * P<0.05; ** P<0.001.
Figure 2. FOXJ2 was predicted to be the target of miR-20a by bioinformatics analysis. (A) Venn diagram of 3 overlapping DEGs from differentially expressed mRNAs and predicted miR-20a target genes. (B, C) GO analysis and KEGG pathway of predicted miR-20a target genes are shown diagrammatically. (D) The heatmap of the predicted miR-20a target genes. Red: high expression level; green: low expression level. (E) The expression level of FOXJ2 was validated according to the GEPIA database. (F) A significant negative correlation was found between FOXJ2 and miR-20a expression.
Figure 3. Foxj2 is a direct target gene of miR-20a in CRC cells. (A) The sequences of miR-20a binding sites within the human Foxj2 3’UTR and schematic reporter constructs. Foxj2-WT represents the reporter constructs containing the entire 3’UTR sequences of Foxj2. Foxj2-MUT represents the reporter constructs containing mutated nucleotides. (B) Analysis of relative luciferase activities of Foxj2-WT and Foxj2-MUT. (C) Western blot analysis showed that overexpression of miR-20a downregulated the Foxj2 protein level in the HCT-116 cells. (D) The expression of Foxj2 protein levels in CRC tissues was established by Western blot (representative results of 5 cases were shown). (E) qPCR analyses of Foxj2 expression in CRC and matched adjacent normal tissues. (F) The relationship between miR-20a and Foxj2 expression was evaluated by Spearman’s correlation analysis of CRC tissues ($R^2=0.476$, $P<0.001$). * $P<0.05$.
that the miR-20a treatment markedly inhibited Foxj2 expression at the protein level, as indicated in Figure 3C.

Additionally, we performed Western blot and qPCR assays to validate the levels of Foxj2 protein and mRNA in 24 freshly collected CRC tissues and para-carcinoma tissues. Foxj2 was found to be markedly downregulated in 18 of the 24 cases, in contrast to the para-carcinoma tissues (P<0.05); Figure 3D shows 5 representative pairs. In comparison with the corresponding para-carcinoma tissues, underexpression of Foxj2 mRNA was observed in the CRC tissues based on qPCR analysis (P<0.05) (Figure 3E). Furthermore, the expression of Foxj2 was negatively correlated with miR-20a expression (Figure 3F).

**Foxj2 partially reversed the effects of miR-20a-mediated CRC progression**

To verify whether miR-20a exerted a biological role in CRC progression via targeting Foxj2, HCT-116 cells were collectively transfected with miR-20a mimics and the Foxj2-overexpressed plasmid. As represented in Figure 4A, the MTT assay indicated that the miR-20a-mediated HCT-116 cell proliferation could be reversed by overexpressed Foxj2. Furthermore, it was indicated in Figure 4B that the upregulated Foxj2 expression partially alleviated the effects of the miR-20a-accelerated S phase and induced cell cycle arrest at the G1 stage. Furthermore, Figure 4C revealed the results of flow cytometry analysis, indicating that the apoptosis rate of HCT-116 cells was inhibited by miR-20a mimics and miR-20a with Foxj2-overexpressed plasmid in contrast to the NC group (P<0.05).

Colonies formation and transwell assays revealed that the promotional roles of miR-20a on cell growth, migration, and invasion of HCT-116 cells could be reversed by the overexpression of Foxj2 (Figure 5A, 5B). It was demonstrated in Figure 5C that the reversion of the impacts of miR-20a in tumor development of HCT-116 cells in vivo. GO and KEGG enrichment analysis results also suggested that the positively regulated EGFR/AKT pathway induced by miR-20a could be inhibited by the overexpression of Foxj2, indicating that the signaling pathway proteins could serve as the downstream regulation factors of Foxj2 (Figure 5D). Overall, our results suggest that the progression of CRC cells was promoted by miR-20a via modulating Foxj2 in vivo and in vitro.

**Discussion**

miR-20a is a member of the mir-17-92 group and is located in the 13q31.1 region. Sequencing data demonstrated that the level of miR-20a was dramatically higher in the CRC cells than in the normal cells, suggesting that miR-20a is positively associated with cell proliferation and colony formation. GO analysis and KEGG pathway analysis showed that Foxj2, as the tumor suppressor gene, targeted miR-20a in CRC. Further research shows that there is a binding site of the 3’ UTR of Foxj2 with the miR-20a+FOXJ2 axis mediates growth and metastasis.
Figure 5. The effects of miR-20a-promoted CRC cell proliferation, migration, and invasion were mediated by the downregulation of Foxj2 in vitro and in vivo. (A) Colony formation assays were used to determine the effects of growth in HCT-116 cells transfected with miR-20a with and without Foxj2 overexpression. The number of colonies was analyzed quantitatively. (B) Cell migration and invasion were performed in HCT-116 cells transfected with miR-20a with and without Foxj2 overexpression. Representative images of migrated and invaded cells (magnification ×100) and quantification of migrated and invaded cells per field. (C) The proliferation activity of xenograft tumors in HCT-116 cells transfected with miR-20a and Foxj2 overexpression on the average volumes in xenograft tumors. (D) The effects of Foxj2 overexpression on the expression of EGFR/AKT signaling pathway proteins in miR-20a-transfected HCT-116 cells were tested by Western blot analysis. * P<0.05.
miR-20a in CRC. Analyses of miR-20a and Foxj2 protein expression levels in human CRC tissues were negatively associated with each other. Flow cytometry analysis showed that the overexpressed Foxj2 does not play a role in apoptosis in vivo. Further, in vitro and in vivo experiments have revealed that overexpression of Foxj2 can effectively reverse the cancer-promoting effects of miR-20a. The above data broadened the horizon for the new regulatory network targeted miR-20a and Foxj2 in CRC, and show the specific mechanism by which miR-20a promotes CRC.

Previous research reported that miR-20a was decreased in several solid tumors, like breast cancer [9] and pancreatic carcinoma [10], but miR-20a is a broadly overexpressed oncogene in various tumors, including anaplastic thyroid cancer [11], lung cancer [12], prostate cancer [13], esophageal squamous cell carcinoma [14], gliomas [15], and colon adenocarcinoma [16], suggesting that the expression and role of miR-20a vary in different tumor types. It has been clearly indicated that the abilities of cell proliferation, invasion, and tumor metastasis in breast cancer cells are suppressed by overexpression of miR-20a [17]. In contrast, miR-20a was observed to inhibit the proliferation and metastasis of the cells of pancreatic carcinoma via directly reducing Stat3 [10]. Other reports indicated that overexpression of miR-20a facilitates the proliferation of ovarian cancer cells and the migration and invasion in the cells of cervical cancer, ovarian cancer and osteosarcoma [18–20], implying that miR-20a acted as the onco-miR. The present study demonstrates that miR-20a is upregulated in CRC tissues, which agrees with the combined detection of TCGA and validation of qPCR. Additionally, miR-20a increased the proliferation and colony-forming ability of CRC cells. Hence, it may be concluded that the deregulation of miR-20a functions as a carcinogenic miRNA and is involved in the progression of CRC. Previous studies showed the crucial effects of miRNAs on the genesis and progression of tumors through regulating the levels of target genes [21]. To verify the bioinformatics analysis for predicting the potential biomarkers of CRC, the screened candidate gene Foxj2 was further validated. Our data also proved that Foxj2 was directly targeted to miR-20a in the CRC cells.

The genes associated with differentiation, apoptosis, proliferation, metabolism, migration, and invasion were reported to be modulated by the Forkhead box (FOX) transcription factor family [22,23]. Forkhead box j2 (Foxj2), belonging to the Forkhead box transcription factor family [24], which exists in several mammals and other vertebrates [25–27], is widely distributed in different organs and tissues of adults and in fetuses. More importantly, recent studies showed that Foxj2 may participate in tumorigenesis [28]. Foxj2 regulates the process of cell migration and cell cycle [29,30]. For example, some observations indicated that elevated Foxj2 inhibits cell migration and invasion of glioma and is positively associated with E-cadherin [31]. In addition, Foxj2 may contribute to the development of EMT via the Notch signaling pathway in non-small cell lung cancer [32]. In the present work, integrated bioinformatics analyses and experiments showed that Foxj2 serves as a tumor inhibitor in CRC. In line with the above results, we found that the migration and invasion of CRC is facilitated by miR-20a via inhibiting Foxj2. These findings show the strong correlation between miR-20a and the Foxj2 axis, which has a significant impact on cell proliferation and tumorigenesis.

Conclusions

This is the first study to demonstrate that CRC tumorigenesis is promoted by targeting of miR-20a with Foxj2. Further results on miR-20a/Foxj2 axis will offer a promising target and open the way for new CRC molecular therapeutics. Nevertheless, it is not clear how Foxj2 influences CRC progression. Hence, further studies are needed to elucidate the effects and functions of Foxj2 in CRC cells.

Conflict of interest

None.
### Supplementary Table 1. Prognostic value of the target genes in CRC patients of the TCGA cohort.

| Gene symbol | Univariate analysis | Multivariate analysis |
|-------------|---------------------|-----------------------|
|             | HR                  | P-value               | HR         | P-value      |
| ZNF133      | 1.537498631         | 0.008697705           | 38         | <0.001      |
| ZKSCAN2     | 1.691982256         | 0.004876581           | 8.9        | <0.001      |
| ZDHHC11     | 1.183004595         | 0.006286627           | 3.2e−01    | <0.001      |
| WSB2        | 0.467109344         | 0.003950621           | 25         | <0.001      |
| WDR86       | 1.40240492          | 0.006044641           | 72         | <0.001      |
| VDAC1       | 0.531016786         | 0.006341117           | 2.7e−03    | <0.001      |
| USH1G       | 1.260619617         | 0.001234788           | 2.1        | <0.001      |
| TRIM25      | 0.573345214         | 0.007816236           | 2.2e−01    | <0.001      |
| TREX2       | 1.373224848         | 0.002772185           | 16         | <0.001      |
| TRABD2B     | 1.285006739         | 0.002930684           | 6.0e−01    | <0.001      |
| TMEM81      | 1.644187505         | 0.008760967           | 1.6e−01    | <0.001      |
| ZKSCAN2     | 1.691982256         | 0.004876581           | 8.9        | <0.001      |
| ZDHHC11     | 1.183004595         | 0.006286627           | 3.2e−01    | <0.001      |
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| VDAC1       | 0.531016786         | 0.006341117           | 2.7e−03    | <0.001      |
| USH1G       | 1.260619617         | 0.001234788           | 2.1        | <0.001      |
| TRIM25      | 0.573345214         | 0.007816236           | 2.2e−01    | <0.001      |
| TREX2       | 1.373224848         | 0.002772185           | 16         | <0.001      |
| TRABD2B     | 1.285006739         | 0.002930684           | 6.0e−01    | <0.001      |
| TMEM81      | 1.644187505         | 0.008760967           | 1.6e−01    | <0.001      |
| TMEM165     | 0.586600114         | 0.007386349           | 1.1e−02    | <0.001      |
| TMCC2       | 1.576746915         | 0.000688545           | 8.9e−02    | <0.001      |
| TINAG       | 0.862994088         | 0.00425559            | 2.9e−01    | <0.001      |
| TGM6        | 1.219997455         | 0.004782644           | 5.1e−01    | <0.001      |
| TCF7L1      | 1.520206387         | 0.00495012            | 5.9        | <0.001      |
| TCEAL5      | 1.213662999         | 0.004119467           | 3.7e−01    | <0.001      |
| TCAIM       | 0.572670505         | 0.003589079           | 1000       | <0.001      |
| SUSD5       | 1.232270257         | 0.003620369           | 5.0e−01    | <0.001      |
| STK32C      | 1.40872781          | 0.005501541           | 8.4e−02    | <0.001      |
| SRPX        | 1.241306378         | 0.001854167           | 2.9        | <0.001      |
| SOX7        | 1.372226734         | 0.003493233           | 1.1e−01    | <0.001      |
| SOWAHA      | 0.738552315         | 0.000853741           | 3.4e−01    | <0.001      |
| SNCB        | 1.457793587         | 1.66509E-05           | 7.1        | <0.001      |
| SLITRK2     | 1.251506902         | 0.001222424           | 5.3e−01    | <0.001      |
| Gene symbol | Univariate analysis |    | Multivariate analysis |    |
|-------------|--------------------|----|----------------------|----|
|             | HR                 | P-value | HR | P-value |
| SLC6A17     | 1.288351213        | 0.00255427 | 6.1e−01 | <0.001 |
| SLC38A8     | 1.288767274        | 0.00473397 | 1.7 | <0.001 |
| SLC28A2     | 0.88596792         | 0.006146815 | 2 | <0.001 |
| SLC20A2     | 0.63283218         | 0.009266251 | 6.6e−02 | <0.001 |
| SLC16A8     | 1.352969047        | 0.0026993 | 2.7e−01 | <0.001 |
| SIX2        | 1.162090077        | 0.01996522  | 2.6 | <0.001 |
| SLC38A8     | 1.288767274        | 0.00473397 | 1.7 | <0.001 |
| SLC38A8     | 1.288767274        | 0.00473397 | 1.7 | <0.001 |
| SLC20A2     | 0.63283218         | 0.009266251 | 6.6e−02 | <0.001 |
| SLC16A8     | 1.352969047        | 0.0026993 | 2.7e−01 | <0.001 |
| SIX2        | 1.162090077        | 0.01996522  | 2.6 | <0.001 |

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| Gene symbol | Univariate analysis | Multivariate analysis |
|-------------|---------------------|-----------------------|
|             | HR                  | P-value               | HR                  | P-value |
| PLEKHG4B    | 1.302343681         | 0.000505889           | 1.1e−01             | <0.001  |
| PIP5K1C     | 1.716568911         | 0.006747328           | 2.6e-01             | <0.001  |
| PDZD4       | 1.17293216          | 0.007277794           | 2.3                 | <0.001  |
| PDE1B       | 1.316236541         | 0.002107239           | 2.6e-01             | <0.001  |
| PDCD6IP     | 0.554647065         | 0.009870352           | 260                 | <0.001  |
| PCDHB8      | 1.143959444         | 0.008713351           | 5.5e-01             | <0.001  |
| PAGE1       | 1.173634426         | 0.004839683           | 2.9e-01             | <0.001  |
| OVOl3       | 1.315953982         | 0.005736431           | 180                 | <0.001  |
| OR2B6       | 1.299546249         | 0.009721983           | 6.9e-01             | <0.001  |
| OAZ2        | 2.842306103         | 0.002254038           | 110                 | <0.001  |
| NXPE9       | 1.434379996         | 0.008906936           | 8.2e-03             | <0.001  |
| NPTXR       | 1.209880744         | 0.002997987           | 3.8e-01             | <0.001  |
| NW1         | 1.585561158         | 0.000736431           | 180                 | <0.001  |
| NPR1        | 1.267838515         | 0.008905722           | 180                 | <0.001  |
| NLGN1       | 1.176341275         | 0.001783991           | 2.5                 | <0.001  |
| NHLRC4      | 1.551869878         | 0.006431943           | 74                  | <0.001  |
| NKG2         | 1.20989542          | 0.001352476           | 140                 | <0.001  |
| NGF         | 1.318819479         | 0.004309054           | 2.5e-01             | <0.001  |
| NfatC1      | 1.307619018         | 0.007989554           | 46                  | <0.001  |
| NECAB2      | 1.27076126          | 0.006135428           | 4.9                 | <0.001  |
| NCKAP5L     | 2.169171268         | 2.56373E-05           | 3.1e-02             | <0.001  |
| NAT1        | 0.73284881          | 0.006734646           | 3.9                 | <0.001  |
| NACAD       | 1.305822623         | 0.00141327            | 48                  | <0.001  |
| MYB         | 0.630652971         | 0.002410283           | 4.9e-01             | <0.001  |
| MAA        | 0.576201489         | 0.001516101           | 2.9                 | <0.001  |
| LLT1L       | 1.34614633          | 0.006077126           | 26                  | <0.001  |
| MRKRN2OS    | 0.654012218         | 0.001024618           | 1.6e-01             | <0.001  |
| METTL9      | 0.538908907         | 0.009335042           | 1.3e-01             | <0.001  |
| MC1R        | 1.29905193          | 0.006969694           | 1.4e-01             | <0.001  |
| MPAKAPK5    | 0.470290813         | 0.00753356            | 490                 | <0.001  |
| MPAK12      | 1.3737705           | 0.00094184            | 18                  | <0.001  |
| MAGEF1      | 1.956272182         | 0.007680343           | 3.4e-04             | <0.001  |
| MAGEA1      | 1.247064893         | 2.7493E-05            | 3.3                 | <0.001  |
| LRP2        | 1.288962734         | 6.10532E-05           | 1.5                 | <0.001  |
| LINGO1      | 1.355302198         | 0.000399767           | 4.6e-01             | <0.001  |
| LEP         | 1.18508016          | 0.002465145           | 12                  | <0.001  |
| MAA        | 0.576201489         | 0.001516101           | 2.9                 | <0.001  |
| MLT11       | 1.34614653          | 0.006077126           | 26                  | <0.001  |
| Gene symbol | Univariate analysis | Multivariate analysis |
|-------------|---------------------|----------------------|
|             | HR                  | P-value              | HR                  | P-value              |
| MKRN2OS     | 0.654012218         | 0.001024618          | 1.6e−01             | <0.001               |
| METTL9      | 0.538908907         | 0.009335042          | 1.3e−01             | <0.001               |
| MC1R        | 1.29905193          | 0.006969694          | 1.4e−01             | <0.001               |
| MAPKAPK5    | 0.470290813         | 0.00753356           | 490                 | <0.001               |
| MAPK12      | 1.3737775           | 0.0094184            | 18                  | <0.001               |
| MAGEF1      | 1.956272182         | 0.007680343          | 3.4e-04             | <0.001               |
| MAGEA1      | 1.247064893         | 2.7493E-05           | 3.3                 | <0.001               |
| LRP2        | 1.288962734         | 6.10532E-05          | 1.5                 | <0.001               |
| LINGO1      | 1.355302198         | 0.00399767           | 4.6e-01             | <0.001               |
| LEP         | 1.18500816          | 0.002465145          | 12                  | <0.001               |
| KCNIP3      | 1.260837142         | 0.004916884          | 4.7e-02             | <0.001               |
| ITIH6       | 1.209106013         | 0.002657523          | 7.3                 | <0.001               |
| ITGAS5      | 1.307298059         | 0.00585555           | 990                 | <0.001               |
| IRX6        | 1.322112702         | 0.00843443           | 3.3e-01             | <0.001               |
| IQSEC3      | 1.27677672          | 0.004666794          | 1.9e-01             | <0.001               |
| INHBB       | 1.17779802          | 0.004605681          | 31                  | <0.001               |
| INHAA       | 1.127695273         | 0.0002343677         | 3.7                 | <0.001               |
| IRX6        | 1.322112702         | 0.00843443           | 3.3e-01             | <0.001               |
| ITGAS5      | 1.307298059         | 0.00585555           | 990                 | <0.001               |
| IRX6        | 1.322112702         | 0.00843443           | 3.3e-01             | <0.001               |
| IQSEC3      | 1.27677672          | 0.004666794          | 1.9e-01             | <0.001               |
| INHBB       | 1.17779802          | 0.004605681          | 31                  | <0.001               |
| INHAA       | 1.127695273         | 0.0002343677         | 3.7                 | <0.001               |
| IRX6        | 1.322112702         | 0.00843443           | 3.3e-01             | <0.001               |
| IQSEC3      | 1.27677672          | 0.004666794          | 1.9e-01             | <0.001               |
| INHBB       | 1.17779802          | 0.004605681          | 31                  | <0.001               |
| INHAA       | 1.127695273         | 0.0002343677         | 3.7                 | <0.001               |
| IRX6        | 1.322112702         | 0.00843443           | 3.3e-01             | <0.001               |
| KPCAL4      | 1.283814727         | 0.00451649           | 6.7                 | <0.001               |
| HOXC6       | 1.181620432         | 0.000133735          | 3.3e-01             | <0.001               |
| HOXC6       | 1.204636727         | 0.000531529          | 2.5                 | <0.001               |
| HEYL        | 1.676211788         | 0.000538317          | 4.5                 | <0.001               |
| HEY1        | 1.587051358         | 0.002290132          | 2.4e-01             | <0.001               |
| HEXB        | 0.461726956         | 0.000952528          | 120000              | <0.001               |
| HDAC7       | 1.96210524          | 0.006035858          | 110000              | <0.001               |
| GSPT1       | 0.489079951         | 0.005568068          | 1.2e-05             | <0.001               |
| GRIK5       | 1.183858849         | 0.008746472          | 5.7e-01             | <0.001               |
| GRID1       | 1.336945729         | 0.00449767           | 3.1                 | <0.001               |
| GPRCSB      | 1.53508947          | 0.000501917          | 13                  | <0.001               |
| GPRASPB1    | 1.272720263         | 0.006263557          | 6.4e-02             | <0.001               |
| GPR156      | 1.308364429         | 0.001153267          | 4.2                 | <0.001               |
| GLYAT1      | 0.860613222         | 0.006528162          | 9.5e-02             | <0.001               |
| GDF15       | 0.833412966         | 0.008604588          | 1.4                 | <0.001               |
| GAREM2      | 1.381779663         | 0.005033009          | 1.6                 | <0.001               |
| GALNT15     | 1.204296146         | 0.008635526          | 1.4e-01             | <0.001               |
| FZD4        | 1.486653675         | 0.006249553          | 3.6e-03             | <0.001               |
| FSTL3       | 1.271624144         | 0.007962522          | 1.6e-01             | <0.001               |
| Gene symbol | Univariate analysis | Multivariate analysis |
|-------------|---------------------|-----------------------|
|             | HR                  | P-value               | HR          | P-value |
| FOXL2NB     | 1.345119444         | 5.37926E-05           | 4.2         | <0.001  |
| FOXL2       | 1.259891809         | 0.000933203           | 6.9e-01     | <0.001  |
| FOXJ2       | 2.472932089         | 0.002849779           | 1.9e-01     | <0.001  |
| FOXD4       | 1.245464377         | 0.005531115           | 3.8         | <0.001  |
| FOXC1       | 1.270115339         | 0.002834468           | 5.4e-01     | <0.001  |
| FNDC5       | 1.332360452         | 0.001709798           | 2.6         | <0.001  |
| FLT3LG      | 1.417336731         | 0.009536758           | 1.8e-02     | <0.001  |
| FES         | 1.443664395         | 0.001757521           | 3.0e-01     | <0.001  |
| FAM117A     | 1.562351037         | 0.001958748           | 400         | <0.001  |
| FAM110D     | 1.383624103         | 0.002865335           | 8.1         | <0.001  |
| FADS1       | 1.287690981         | 0.00690719            | 1.9         | <0.001  |
| FABP4       | 1.13959679          | 0.001160698           | 4.1e-01     | <0.001  |
| ETR1        | 1.265980039         | 0.005035667           | 1.0e-01     | <0.001  |
| ETS2        | 0.668284016         | 0.005916623           | 7.1         | <0.001  |
| ERMAP       | 0.568400341         | 0.009071429           | 2.3e-02     | <0.001  |
| ERICH3      | 1.23067448          | 0.006719883           | 1.9         | <0.001  |
| EREG        | 0.887017859         | 0.006138749           | 15          | <0.001  |
| EPS8        | 0.583792225         | 0.00678897            | 2.8         | <0.001  |
| ERO1        | 1.37759357          | 0.00865002            | 6.7         | <0.001  |
| EPB4        | 0.722678923         | 0.00896647            | 35          | <0.001  |
| EMD1        | 0.744888852         | 0.000584074           | 36          | <0.001  |
| EFF2S3B     | 0.87869205          | 0.003802776           | 5.2e-01     | <0.001  |
| EPHD1       | 1.39062874          | 0.001678372           | 5           | <0.001  |
| EBF4        | 1.460934784         | 0.000259432           | 4.2         | <0.001  |
| DRD4        | 1.302006231         | 0.004539947           | 60          | <0.001  |
| DOCK3       | 1.289854413         | 0.002209175           | 3.0e-01     | <0.001  |
| DMPK        | 1.427657686         | 0.002350812           | 4.0e-01     | <0.001  |
| DLG4        | 1.479923277         | 0.00994533            | 1.1e-01     | <0.001  |
| DHX15       | 0.392830659         | 0.00995095            | 2400        | <0.001  |
| DCTN1       | 2.095513206         | 0.007449317           | 1.1e-05     | <0.001  |
| CYP4F12     | 0.741142308         | 0.000753205           | 3.7         | <0.001  |
| CTNNA1      | 0.519488852         | 0.000584074           | 36          | <0.001  |
| CTF1        | 1.248947118         | 0.007277252           | 7.2e-01     | <0.001  |
| EPHB2       | 0.722678923         | 0.00896647            | 35          | <0.001  |
| CT4SA1      | 1.285770132         | 0.0081524             | 4.3e-02     | <0.001  |
| CRY2        | 1.862665669         | 0.003229067           | 2.9e-02     | <0.001  |
| CRISPLD2    | 1.347498521         | 0.00831665            | 1.9e-01     | <0.001  |
| CORO6       | 1.314726218         | 0.002462567           | 2.1e-01     | <0.001  |
| Gene symbol | Univariate analysis | Multivariate analysis |
|------------|---------------------|----------------------|
|            | HR                  | P-value              | HR                  | P-value              |
| CNTNAP1    | 1.297543992         | 0.008474966         | 3.0e−01             | <0.001               |
| CNPY1      | 1.279382847         | 0.005932603         | 2.4                 | <0.001               |
| CLSTN3     | 1.648521955         | 0.004361652         | 68                  | <0.001               |
| CLRN3      | 0.690451251         | 0.000104942         | 18                  | <0.001               |
| CILP       | 1.116653758         | 0.00881835          | 4.6e−01             | <0.001               |
| CIDEA      | 1.157068648         | 0.004817007         | 2.2                 | <0.001               |
| CHDH       | 0.720216727         | 0.008461958         | 3.3e−01             | <0.001               |
| CHD5       | 1.36465549          | 0.000340142         | 1.6                 | <0.001               |
| CHD3       | 1.568155448         | 0.007182613         | 4.3                 | <0.001               |
| CERS1      | 1.256482733         | 0.00187944          | 2.5e−01             | <0.001               |
| CELF4      | 1.266315192         | 0.000502411         | 32                  | <0.001               |
| CEACAM6    | 0.830447969         | 0.00681502          | 4.4                 | <0.001               |
| CDS1       | 0.619434738         | 0.00282094          | 3.2e−01             | <0.001               |
| CDHR2      | 0.847514909         | 0.003798721         | 2.9                 | <0.001               |
| CDH17      | 0.729770539         | 0.000635009         | 1.4e−01             | <0.001               |
| CDC25C     | 0.684151197         | 0.00688226          | 8.2                 | <0.001               |
| CCR1       | 0.677114592         | 0.00178477          | 5.3e−01             | <0.001               |
| CCNA2      | 0.676285748         | 0.00597114          | 7.3e−04             | <0.001               |
| CAVIN1     | 1.354099446         | 0.00433824          | 2.7                 | <0.001               |
| CATSPERG   | 1.370530111         | 0.007056119         | 3                   | <0.001               |
| CARKS1     | 1.264766074         | 0.0069264           | 1.5                 | <0.001               |
| CADPS      | 0.867620712         | 0.008019302         | 1.8                 | <0.001               |
| C7         | 1.154533485         | 0.00912349          | 11                  | <0.001               |
| C4orf50    | 1.306276285         | 0.009847324         | 2.2                 | <0.001               |
| C1QL4      | 1.265743223         | 0.006675407         | 3.9e−01             | <0.001               |
| C19orf81   | 1.223741881         | 0.004326188         | 5.9e−01             | <0.001               |
| C10orf96   | 1.331680254         | 0.00549506          | 3.9e−01             | <0.001               |
| BST2       | 1.245735057         | 0.002990982         | 4.0e−01             | <0.001               |
| BHMG1      | 1.326825141         | 0.000145561         | 7.0e−01             | <0.001               |
| AXIN2      | 0.814861386         | 0.00393369          | 2.6e−01             | <0.001               |
| ATP6V1B1   | 1.39786657         | 1.29412E-05         | 1.5e−01             | <0.001               |
| ATP2A1     | 1.364043136         | 0.001518394         | 15                  | <0.001               |
| ASPG       | 0.828092956         | 0.002850215         | 4.1e−01             | <0.001               |
| ARV1       | 0.645960387         | 0.006296874         | 160                 | <0.001               |
| ARHGEF17   | 1.47498475          | 0.00307932          | 5                   | <0.001               |
| APELA      | 0.81227505          | 0.00676816          | 5.2e−01             | <0.001               |
| APC2       | 1.595680863         | 0.0064022           | 61                  | <0.001               |
| AOC2       | 1.319226342         | 0.003690611         | 1.9e−01             | <0.001               |
| Gene symbol | Univariate analysis | Multivariate analysis |
|-------------|---------------------|----------------------|
|             | HR                  | P-value              | HR                  | P-value              |
| ANKS4B      | 0.657463731         | 0.000561344          | 6.7e-02             | <0.001               |
| ANKR2D2     | 0.730856239         | 0.002877536          | 2.1                 | <0.001               |
| ANAPC5      | 0.4607465           | 0.0094694            | 7.8e-03             | <0.001               |
| AIF1L       | 1.281527978         | 0.003975246          | 14                  | <0.001               |
| AGMAT       | 0.701313746         | 0.00415433           | 10                  | <0.001               |
| AGFG1       | 0.471524071         | 0.008488669          | 9.8e-04             | <0.001               |
| ADARB1      | 1.742317631         | 0.00124682           | 1.3e-01             | <0.001               |
| ADAM9       | 0.686991601         | 0.00312403           | 100                 | <0.001               |
| ADAM11      | 1.388217427         | 0.00336073           | 3.8e-01             | <0.001               |
| ACOT11      | 0.679141782         | 0.004708273          | 9.4                 | <0.001               |
| AC129492.1  | 1.368845979         | 0.002649713          | 4.9e-02             | <0.001               |
| AC009336.2  | 1.302764599         | 0.00840112           | 5.8e-01             | <0.001               |
| CD1A        | 0.761549955         | 0.002279799          | 7.2e-01             | <0.001               |
| STARD9      | 1.264169741         | 0.008620268          | 1.4                 | 0.002                |
| DAPK1       | 1.249692788         | 0.003244655          | 1.3                 | 0.002                |
| DSG4        | 0.782143447         | 0.002206743          | 1.3                 | 0.003                |
| TMEM88      | 1.561816138         | 0.000335998          | 6.6e-01             | <0.001               |
| FAM151B     | 0.623891263         | 0.00218039           | 6.1e-01             | 0.005                |
| SYCE2       | 1.308137768         | 0.004935619          | 7.1e-01             | 0.007                |
| JPH3        | 1.233591685         | 0.00449381           | 8.0e-01             | 0.008                |
| JDP2        | 1.704624037         | 0.00979684           | 5.3e-01             | 0.011                |
| ZDHHC3      | 0.40145391          | 0.000119984          | 2.2                 | 0.015                |
| CCL24       | 0.857409516         | 0.00518276           | 8.5e-01             | 0.015                |
| ALDOB       | 0.884387033         | 0.009297044          | 1.1                 | 0.019                |
| RIMK1B      | 1.264805716         | 0.009187316          | 7.6e-01             | 0.022                |
| C10orf55    | 0.753029457         | 0.008084309          | 1.3                 | 0.023                |
| F2RL2       | 0.82236627          | 0.00801463           | 8.3e-01             | 0.042                |
| ANXA3       | 0.767488008         | 0.002455007          | 8.0e-01             | 0.045                |
| ALPP        | 1.143185335         | 0.007108907          | 1.1                 | 0.045                |

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