Molecular Pathogenesis of Colorectal Cancer: Impact of Oncogenic Targets Regulated by Tumor Suppressive miR-139-3p

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Abstract: We recently determined the RNA sequencing-based microRNA (miRNA) expression signature of colorectal cancer (CRC). Analysis of the signature showed that the expression of both strands of pre-miR-139 (miR-139-5p, the guide strand, and miR-139-3p, the passenger strand) was significantly reduced in CRC tissues. Transient transfection assays revealed that expression of miR-139-3p blocked cancer cell malignant transformation (e.g., cell proliferation, migration, and invasion). Notably, expression of miR-139-3p markedly blocked RAC-alpha serine/threonine-protein kinase (AKT) phosphorylation in CRC cells. A combination of in silico database and gene expression analyses of miR-139-3p-transfected cells revealed 29 putative targets regulated by miR-139-3p in CRC cells. RNA immunoprecipitation analysis using an Argonaute2 (AGO2) antibody revealed that KRT80 was efficiently incorporated into the RNA-induced silencing complex. Aberrant expression of Keratin 80 (KRT80) was detected in CRC clinical specimens by immunostaining. A knockdown assay using small interfering RNA (siRNA) targeting KRT80 showed that reducing KRT80 expression suppressed the malignant transformation (cancer cell migration and invasion) of CRC cells. Importantly, inhibiting KRT80 expression reduced AKT phosphorylation in CRC cells. Moreover, hexokinase-2 (HK2) expression was reduced in cells transfected with the KRT80 siRNAs or miR-139-3p. The involvement of miRNA passenger strands (e.g., miR-139-3p) in CRC cells is a new concept in miRNA studies. Our tumor-suppressive miRNA-based approach helps elucidate the molecular pathogenesis of CRC.

Keywords: microRNA; expression signature; miR-139-3p; tumor suppressor; colorectal cancer; KRT80; HK2; AKT

1. Introduction

According to the World Health Organization (Globocan 2020), colorectal cancer (CRC) is the third most common cancer (over 1,800,000 cases) worldwide and the second leading cause of cancer-related deaths (over 880,000 deaths) [1]. In clinical practice, the prognosis of CRC is relatively good if diagnosed early. However, the prognosis is consistently poor in advanced cases, with a 5-year survival rate of approximately 14% (stage III or stage IV metastatic disease) [2]. At the time of the initial diagnosis, approximately 14–18% of patients with CRC have metastases, and the treatment strategies for unresectable cases are limited [3].

The oncogenesis of CRC is illustrated by a well-known multistep model of cancer cells [4–6]. From previous studies, mutations in various genes involved in the oncogenesis of CRC (e.g., APC, TP53, SMAD4, KRAS, and PIK3CA) and activation of cancer signaling pathways (e.g., WNT, RAS/MAPK, PI3K, TGF-β, P53, and DNA mismatch-repair) caused by these gene mutations have been reported [4]. CRC cells have highly heterogeneous properties, requiring new therapeutic parameters for CRC from genetic and genomic points of view. As a result of this molecular heterogeneity, recent genome-wide transcriptome
analyses have revealed that CRC cells can be molecularly classified into four consensus molecular subtypes (CMS1 to CMS4) [7]. The future treatment strategies for CRC patients will be based on these subtypes.

As a result of the Human Genome Project, it has become clear that a vast number of functional non-coding RNA molecules (ncRNAs) are present in the human genome [8]. Current studies have shown that numerous ncRNAs play important roles in various biological activities such as the stabilization of RNA molecules and regulation of gene expression and the cell cycle [9,10]. Extensive research to date has revealed that ncRNA dysregulation is deeply involved in the initiation and development of human diseases, including cancer [11].

Among ncRNAs, microRNAs (miRNAs), consisting of only 19–22 nucleotides, have been well studied in cancer research fields. They function as fine-tuners of gene expression control in a sequence-dependent manner [12]. A single miRNA controls numerous genes, and in turn, a single gene is controlled by numerous miRNAs [13]. As a result, miRNAs and their target genes form a very complex network within cells, and it is easy to imagine that aberrant expression of miRNAs disrupts this RNA network. Many studies have shown aberrant expression of miRNAs in CRC cells, and these miRNAs act as oncogenes and/or tumor suppressors by targeting cancer-related genes in CRC cells [14–16].

More recently, to identify aberrantly expressed miRNAs in CRC cells, we determined the miRNA expression signature of CRC by RNA sequencing [17]. Our signature revealed that both the guide and passenger strands of 16 miRNAs (e.g., miR-9, miR-28, miR-29c, miR-30a, miR-99a, miR-100, miR-125b, miR-129, miR-133a, miR-139, miR-143, miR-145, miR-218, miR-195, miR-490, and miR-497) derived from pre-miRNAs were downregulated [17]. Our recent studies showed that some passenger strands of miRNAs (e.g., miR-30a, miR-99a, miR-143, miR-145, and miR-490) act as tumor-suppressive miRNAs in a wide range of cancers [17–21]. Interestingly, the genes regulated by a specific miRNA differ depending on the type of cancer.

In this study, we focused on miR-139-3p (the passenger strand of pre-miR-139) and investigated its functional significance and target oncogenes in CRC cells. Notably, ectopic expression of miR-139-3p markedly blocked the phosphorylation of RAC-alpha serine/threonine-protein kinase (AKT) in CRC cells. Our search strategy for miRNA targets revealed a total of 29 genes as putative candidate targets of miR-139-3p in CRC cells. Of these, keratin 80 (KRT80) was found to be a direct target of miR-139-3p, and its aberrant expression enhanced the malignant transformation of CRC cells. Involvement of the passenger strand of miRNA and its gene targets in CRC pathogenesis is a new concept and provides novel insights into the molecular pathogenesis of CRC.

2. Results

2.1. Expression of miR-139-5p and miR-139-3p in CRC Specimens

Recently, we determined the miRNA expression signature of CRC by miRNA sequencing using CRC clinical specimens (GEO accession number: GSE183437). Analysis of the signature showed that 84 miRNAs were upregulated, and 70 were downregulated in CRC tissues (Figure 1A). Among downregulated miRNAs in CRC tissues, we focused on miR-139-5p (the guide strand) and miR-139-3p (the passenger strand), because both strands of miRNAs derived from pre-miR-139 were significantly downregulated in CRC tissues. Our interest is to clarify how the passenger strand of miRNA is involved in the malignant transformation of CRC cells. The mature sequences of the two microRNAs are shown in Figure 1B.
CRC tissues and noncancerous tumor-adjacent tissues (27 paired) were used to verify the expression status of miR-139-5p, miR-139-3p, and their target genes. Clinical information of these specimens is shown in Table S1. The expression levels of miR-139-5p (p < 0.001) and miR-139-3p (p < 0.001) were significantly lower in CRC tissues than normal tissues (Figure 1C). Next, we examined the expression levels of miR-139-5p and miR-139-3p in two CRC cell lines, HCT116 and DLD-1. In these cell lines, the expression levels of miR-139-5p and miR-139-3p were lower than those in normal epithelial tissues (Figure 1C).

Furthermore, a positive correlation was detected between miR-139-5p and miR-139-3p expression levels by Spearman’s rank analysis (r = 0.559, p < 0.001; Figure 1D).

2.2. Ectopic Expression Assays of miR-139-5p and miR-139-3p in CRC Cell Lines

To investigate the tumor-suppressive functions of miR-139-5p and miR-139-3p, we ectopically expressed mature miR-139-5p and miR-139-3p in two CRC cell lines, HCT116...
and DLD-1, and performed functional assays, e.g., cancer cell proliferation, migration, and invasion. After miR-139-5p transfection, cancer cell migration in both cell lines was significantly inhibited (Figure 2A–C). In contrast, the malignant phenotypes of cancer cells, e.g., proliferation, migration, and invasion, were significantly reduced by miR-139-3p transfection in both cell lines (Figure 2A–C). Representative images from the migration and invasion assays are shown in Figure S1.

Figure 2. Tumor-suppressive functions of miR-139-5p and miR-139-3p in CRC cells (HCT116 and DLD-1). (A) Cell proliferation assessed by XTT assay. At 72 h after transient transfection of miRNAs, cancer cell viability was analyzed. (B) Cell migration ability assessed using a membrane culture system. At 48 h after miRNA transfection, the cells were seeded into the migration chambers. (C) Cell invasion ability assessed by Matrigel invasion assay. At 48 h after miRNA transfection, the cells were seeded into the invasion chambers. (N.S.: not significant compared to mock group.).

2.3. Identification of Oncogenes Regulated by miR-139-3p in CRC Cells

Based on these expressions and functional analysis, aberrant expression of miR-139-3p and disruption of its gene regulation mechanisms were considered to be more deeply involved in the malignant pathogenesis of CRC. We focused on miR-139-3p (passenger strand) for further validation.

We investigated whether epigenetic modifications affect the downregulation of miR-139-3p in CRC cells. After treatment of Trichostatin A (TSA) in CRC cells, the expression level of miR-139 was increased compared to TSA untreated cells (Figure S2A). In addition, miR-139-3p expression level was elevated by 5-aza-2-deoxycytidine (5-aza-dC) treatment in CRC cells (Figure S2B). These results suggest that histone deacetylation and DNA methylation are closely involved in the downregulation of miR-139-3p in CRC cells.

The following hypotheses regarding miR-139-3p target genes in CRC cells were made: the target genes of miR-139-3p have one or more binding site(s), are downregulated after miR-139-3p transfection in CRC cells, and are upregulated in CRC tissues. We combined the
gene expression data from two databases (TargetScan and GEPIA2) with gene expression data from miR-139-3p-transfected CRC cells (GSE155659) to search for genes that meet these three criteria. A flowchart of the search strategy is shown in Figure 3. A total of 95 putative targets of miR-139-3p in CRC cells were identified.

![Flowchart of the search strategy for oncogenes regulated by miR-139-3p in CRC cells.](image)

**Figure 3.** Search strategy for oncogenes regulated by miR-139-3p in CRC cells. To identify miR-139-3p targets in CRC cells, we assessed the TargetScan database and gene expression data from miR-139-3p-transfected HCT116 cells (GEO accession number: GSE155659). To evaluate genes upregulated in CRC clinical specimens, we used the GEPIA2 database. A total of 29 genes were identified as potential oncogenic targets regulated by miR-139-3p in CRC cells.

We assessed the expression levels of putative miR-139-3p target genes in CRC clinical tissues using The Cancer Genome Atlas database via the GEPIA2 platform. A total of 29 genes were significantly upregulated in CRC clinical specimens (colon adenocarcinoma or rectal adenocarcinoma) in this database (p < 0.01; Table 1, Figure S3). GEPIA2 analysis revealed that the expression level of KRT80 was fairly low in normal tissues (Figure S3). Genes expressed exclusively in cancer cells are appropriate therapeutic targets for CRC. We focused on KRT80 in the subsequent functional analyses in CRC cells.

**Table 1.** Candidate gene targets of miR-139-3p significantly overexpressed in CRC cells.

| Entrez Gene ID | Gene Symbol | Gene Name | No. of Binding Sites | log2 FC miR-139-3p-Transfected HCT116 Cells |
|---------------|-------------|-----------|---------------------|---------------------------------------------|
| 9768          | KIAA0101    | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 | 2  | -3.773 |
| 6541          | SLC7A1      | KIAA0101 | 4  | -3.072 |
| 23094         | SIPA1L3     | SH3 domain and tetratricopeptide repeats 2 | 2  | -2.961 |
| 79628         | SH3TC2      | SH3 domain and tetratricopeptide repeats 2 | 1  | -2.921 |
| 201232        | SLC16A13    | Solute carrier family 16, member 13 | 1  | -2.905 |
| 27286         | SRRX2       | Sushi-repeat containing protein, X-linked 2 | 1  | -2.901 |
| 118932        | ANKRD22     | Ankyrin repeat domain 22 | 1  | -2.901 |
| 3099          | HK2         | Hexokinase 2 | 1  | -2.729 |
| 57116         | ZNF695      | Zinc finger protein 695 | 2  | -2.525 |
| 3352          | HTR1D       | 5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled | 1  | -2.510 |
| 140893        | RBP8NL      | RBP8 N-terminal like | 1  | -2.509 |
| 4171          | MCM2        | Minichromosome maintenance complex component 2 | 1  | -2.475 |
| 201266        | SLC39A11    | Solute carrier family 39, member 11 | 1  | -2.474 |
2.4. Direct Regulation of KRT80 by miR-139-3p in CRC Cells

In CRC cells transfected with miR-139-3p, both the mRNA and protein levels of KRT80 were significantly downregulated (Figure 4A).

![Figure 4. Regulation of KRT80 expression by miR-139-3p in CRC cells. (A) Reduced mRNA and protein levels of KRT80 in miR-139-3p-transfected CRC cells. At 72 h after miR-139-3p transfection, the cells were subjected to real-time PCR and Western blot analyses. (B) RNA immunoprecipitation assay of RISC-incorporated KRT80 mRNA using an Ago2 antibody. Real-time PCR data indicated that KRT80 mRNA was incorporated into RISC. Schematic illustration showed miR-139-3p and KRT80 were incorporated into RISC. (C) TargetScan database analysis of the putative miR-139-3p binding site in the 3’UTR of KRT80. Dual-luciferase reporter assays showed reduced luminescence activity after co-transfection of the wild-type vector and miR-139-3p in HCT116 cells (left panel). Normalized data were calculated as the Renilla/Firefly luciferase activity ratio (N.S.: not significant compared with the mock group).](image-url)

### Table 1. Cont.

| Entrez Gene ID | Gene Symbol | Gene Name                                      | No. of Binding Sites | miR-139-3p-Transfected HCT116 Cells log2 FC | C | HCT116 Cells | DLD-1 | C | HCT116 Cells |
|---------------|-------------|------------------------------------------------|----------------------|--------------------------------------------|--|--|---------------|-------|---|---------------|
| 90361         | HN1L        | Hematological and neurological expressed 1-like | 1                    | −2.441                                     |   |               |       |   |               |
| 57402         | S100A14     | S100 calcium binding protein A14               | 1                    | −2.394                                     |   |               |       |   |               |
| 8884          | SLC5A6      | Solute carrier family 5 (sodium/multivitamin and iodide cotransporter), member 6 | 1                    | −2.371                                     |   |               |       |   |               |
| 55612         | FERMT1      | Fermitin family member 1                       | 1                    | −2.359                                     |   |               |       |   |               |
| 9721          | GPRIN2      | G protein regulated inducer of neurite outgrowth 2 | 1                    | −2.358                                     |   |               |       |   |               |
| 54552         | GNL3L       | Guanine nucleotide binding protein-like 3      | 1                    | −2.252                                     |   |               |       |   |               |
| 5653          | KLK6        | Kallikrein-related peptidase 6                 | 1                    | −2.251                                     |   |               |       |   |               |
| 157285        | SGK223      | Tyrosine-protein kinase sgk223                 | 1                    | −2.224                                     |   |               |       |   |               |
| 144501        | KRT80       | Keratin 80                                     | 1                    | −2.193                                     |   |               |       |   |               |
| 154796        | AMOT        | Angiomotin                                      | 1                    | −2.126                                     |   |               |       |   |               |
| 9052          | GPRC5A      | G protein-coupled receptor, family C, group 5, member A | 1                    | −2.114                                     |   |               |       |   |               |
| 54815         | GATAD2A     | GATA zinc finger domain containing 2A           | 1                    | −2.113                                     |   |               |       |   |               |
| 3898          | LAD1        | Ladinin 1                                      | 1                    | −2.046                                     |   |               |       |   |               |
| 414           | ARSD        | Arylsulfatase D                                | 1                    | −2.023                                     |   |               |       |   |               |
| 90381         | TICRR       | TOPBP1-interacting checkpoint and replication regulator | 1                    | −2.022                                     |   |               |       |   |               |
| 10189         | ALYREF      | Aly/REF export factor                          | 1                    | −2.006                                     |   |               |       |   |               |

FC: fold change.
Next, RNA immunoprecipitation (RIP) analysis was performed to confirm that \textit{KRT80} mRNA was incorporated into the RNA-induced silencing complex (RISC) after \textit{miR-139-3p} transfection. The RIP assay concept is illustrated in a schematic in Figure 4B. In samples subjected to immunoprecipitation using an Argonaute2 (AGO2) antibody, quantitative real-time reverse-transcription PCR (qRT-PCR) showed that the \textit{KRT80} mRNA level was significantly higher than that in mock and miRNA control-transfected cells \((p < 0.001; \text{Figure } 4B)\). Ago2-bound \textit{miR-139-3p} and \textit{KRT80} mRNA were isolated by immunoprecipitation using the AGO2 antibody, suggesting that the RISC plays a central role in miRNA biogenesis (Figure 4B).

Finally, a dual-luciferase reporter assay was performed to confirm that \textit{miR-139-3p} binds directly to the 3′ untranslated regions (UTR) of \textit{KRT80}. Luciferase activity was significantly reduced following co-transfection with \textit{miR-139-3p} and a vector containing the \textit{miR-139-3p}-binding site within the 3′-UTR of \textit{KRT80} (Figure 4C). In contrast, co-transfection with a vector containing the \textit{KRT80} 3′-UTR in which the \textit{miR-139-3p}-binding site was deleted resulted in no change in luciferase activity (Figure 4C).

### 2.5. Knockdown Assays by Small Interfering RNAs (siRNAs) Targeting \textit{KRT80} in CRC Cell Lines

To assess the functional significance of \textit{KRT80} in CRC cells, we performed knockdown assays using siRNAs corresponding to \textit{KRT80} mRNA. First, the inhibitory effects of two different siRNAs (\textit{siKRT80}-1 and \textit{siKRT80}-2) targeting \textit{KRT80} in two cell lines were examined. Both \textit{KRT80} mRNA and protein levels were effectively suppressed after transfection of each siRNA into HCT116 and DLD-1 cells (Figure S4).

Knockdown of \textit{KRT80} slightly inhibited cell proliferation (Figure 5A) and markedly inhibited migration and invasion in both HCT116 and DLD-1 cells (Figure 5B,C). Representative photographs from the migration and invasion assays are shown in Figure S5.

**Figure 5.** Functional assays in CRC cells after siRNA-mediated \textit{KRT80} knockdown. (A) Cell proliferation assessed by XTT assay at 72 h after siRNA transfection. (B) Cell migration assessed using a membrane culture system at 48 h after seeding miRNA-transfected cells into the chambers. (C) Cell invasion assessed by Matrigel invasion assays at 48 h after seeding miRNA-transfected cells into the chambers. (D) Western blot analysis of AKT and phosphorylated AKT at 72 h after \textit{siKRT80} and \textit{miR-139-3p} transfection.
Based on the previous report that overexpression of KRT80 induced epithelial-mesenchymal transition (EMT)-related genes and activated AKT signaling via phosphorylation of AKT (Ser 473) [22], Western blotting for phosphorylation of AKT was performed on KRT80 and miR-139-3p.

Notably, transfection of the KRT80 siRNAs suppressed the phosphorylation of AKT (Figure 5D).

In addition, expression of miR-139-3p markedly inhibited the phosphorylation of AKT in CRC cells, according to Western blot analysis.

2.6. Aberrant Expression of KRT80 Protein in CRC Clinical Specimens

Protein expression of KRT80 was assessed by immunohistochemistry in CRC clinical specimens. Overexpression of KRT80 protein was detected in cancer lesions (Figure 6).

![Figure 6](image_url)

Figure 6. Immunohistochemical staining of KRT80 in CRC clinical specimens. Hematoxylin and eosin (HE) staining and KRT80 immunohistochemical staining in two colorectal cancer patients: (A) female aged 75 years with stage IV CRC, (B) male aged 82 years with stage IIIC CRC. The slides on the left show HE staining, and those on the right side show KRT80 immunohistochemical staining at 40× (upper) and 200× (lower) magnifications. KRT80 immunohistochemical staining showed that staining was confined to cancer tissues, with no staining in the stroma or mucus components.

2.7. KRT80-Mediated RNA Networks in CRC Cells

To explore KRT80-regulated RNA networks in CRC, we performed comprehensive gene expression analyses in KRT80-knockdown CRC cells. A total of 52 genes were identified as downregulated in both KRT80-knockdown CRC cell lines (log2 fold change < −1.0: Table 2). Our expression data were deposited in the GEO database (GEO accession number: GSE208785).

In this study, we focused on hexokinase 2 (HK2) because it was identified as a miR-139-3p target in CRC cells (Table 1). HK2 was commonly regulated by miR-139-3p and KRT80 in CRC cells (Figure 7A). Moreover, HK2 was directly regulated by miR-139-3p in CRC cells, by RIP assay and dual luciferase reporter assay (Figure S6). In addition, HK2 expression was upregulated in CRC tissues (Figure S3), and a vast number of studies showed that aberrant expression of HK2 enhances cancer cell malignant transformation in various types of cancers. Our results showed that HK2 expression was reduced in cells transfected with siKRT80 (Figure 7B) or miR-139-3p (Figure 7C).
| Entrez Gene ID | Gene Symbol | Gene Name | siKRT80-1-Transfected HCT116 Cells log2 FC < -1 | siKRT80-2-Transfected HCT116 Cells log2 FC < -1 |
|---------------|-------------|-----------|---------------------------------------------|---------------------------------------------|
| 4155          | MBP         | Myelin basic protein 7 | -4.259 | -3.823 |
| 5027          | P2RX7       | Purinergic receptor P2X 7 | -4.078 | -1.151 |
| 7274          | TTPA        | Alpha tocopherol transfer protein | -2.983 | -1.257 |
| 51399         | DACT1       | Disheveled binding antagonist of beta catenin 1 | -2.745 | -1.248 |
| 5163          | PDK1        | Pyruvate dehydrogenase kinase 1 | -2.686 | -2.183 |
| 114088        | TRIM9       | Tripartite motif containing 9 | -2.641 | -1.383 |
| 54434         | SSH1        | Slingshot protein phosphatase 1 | -2.465 | -1.668 |
| 148418        | SAMD13      | Sterile alpha motif domain containing 13 | -2.444 | -1.292 |
| 284716        | RIMKLA      | Ribosomal modification protein rim like family member A | -2.323 | -1.662 |
| 144501        | KRT80       | Keratin 80 | -2.321 | -1.878 |
| 285735        | LINC00326   | Long intergenic non-protein coding RNA 326 | -2.257 | -1.868 |
| 9194          | SLC16A7     | Solute carrier family 16 member 7 | -2.221 | -1.022 |
| 112399        | EGLN3       | egl-9 family hypoxia inducible factor 3 | -2.209 | -1.350 |
| 256435        | ST6GALNAC3  | ST6 N-acetylglactosaminide alpha-2,6-sialyltransferase 3 | -2.185 | -1.875 |
| 4907          | NT5E        | S’-nucleotidase ecto | -2.087 | -1.564 |
| 100287314     | LINC00941   | Long intergenic non-protein coding RNA 941 | -2.077 | -1.532 |
| 254128        | NIFK-AS1    | NIFK antisense RNA 1 | -2.049 | -1.204 |
| 3099          | HK2         | Hexokinase 2 | -1.916 | -3.518 |
| 1956          | EGFR        | Epidermal growth factor receptor | -1.872 | -1.096 |
| 7378          | UPP1        | Uridine phosphorylase 1 | -1.854 | -1.023 |
| 51384         | WNT16       | Wnt family member 16 | -1.835 | -1.799 |
| 115330        | GPR146      | G protein-coupled receptor 146 | -1.827 | -1.710 |
| 170384        | FUTT1       | Fucosyltransferase 11 | -1.818 | -1.129 |
| 2113          | ETS1        | ETS proto-oncogene 1, transcription factor | -1.754 | -1.284 |
| 861           | RUNX1       | RUNX family transcription factor 1 | -1.720 | -1.209 |
| 10397         | NDRG1       | N-myc downstream regulated 1 | -1.666 | -1.856 |
| 22989         | MYH15       | Myosin heavy chain 15 | -1.634 | -1.744 |
| N.A.          | Inc-ORI01H1-1 | Inc-ORI01H1-1 | -1.576 | -1.277 |
| 644316        | FLJ43315    | Asparagine synthetase pseudogene | -1.568 | -1.405 |
| 4781          | NFIB        | Nuclear factor I b | -1.565 | -1.333 |
| 25886         | POC1A       | POC1 centriolar protein A | -1.549 | -1.338 |
| N.A.          | Inc-CLEC2D-7 | Inc-CLEC2D-7 | -1.447 | -1.571 |
| 51175         | TUBE1       | Tubulin epsilon 1 | -1.383 | -1.488 |
| 10549         | PRDX4       | Peroxiredoxin 4 | -1.373 | -1.158 |
| 843           | CASP10      | Caspase 10 | -1.347 | -1.054 |
| 3613          | IMPA2       | Inositol monophosphatase 2 | -1.320 | -1.712 |
| 100505933     | ADD3-AS1    | ADD3 antisense RNA 1 | -1.296 | -1.095 |
| 4783          | NFIL3       | Nuclear factor, interleukin 3 regulated | -1.267 | -1.184 |
| 57834         | CYP4F11     | Cytochrome P450 family 4 subfamily F member 11 | -1.265 | -1.522 |
| 11199         | ANXA10      | Annexin A10 | -1.262 | -2.649 |
| 64946         | CENPH       | Centromere protein H | -1.251 | -1.236 |
| 8614          | STC2        | Stanniocalcin 2 | -1.243 | -1.373 |
| 286144        | TRIMK       | TRIMK motif containing | -1.194 | -1.351 |
| 2035          | EPB41       | Erythrocyte membrane protein band 4.1 | -1.167 | -1.156 |
| 28996         | HIPK2       | Homeodomain interacting protein kinase 2 | -1.138 | -1.166 |
| 4233          | MET         | homeodoym domain interacting protein kinase | -1.130 | -1.247 |
| 100506211     | MIR210HG    | MIR210 host gene | -1.109 | -1.795 |
| 23015         | GOLGA8A     | Golgin A8 family member A | -1.068 | -1.087 |
| 23516         | SLC39A14    | Solute carrier family 39 member 14 | -1.063 | -1.441 |
| 84986         | ARHGAP19    | Rho gtpase activating protein 19 | -1.062 | -1.035 |
| 255082        | CASC2       | Cancer susceptibility 2 | -1.045 | -1.546 |
| 100507065     | LOC100507065 | Uncharacterized LOC100507065 | -1.032 | -1.425 |

FC: fold change. N.A.: not available.
Figure 7. Regulation of HK2 expression by miR-139-3p and KRT80 in CRC cells. (A) Identification of genes commonly regulated by miR-139-3p and KRT80 in CRC cells. (B) Reduced expression of HK2 after miR-139-3p transfection in CRC cells (HCT116 and DLD-1). At 72 h after miRNA transfection, the cells were subjected to real-time PCR. (C) Reduced expression of HK2 after transfection of siRNAs targeting KRT80 in CRC cells (HCT116 and DLD-1). At 72 h after siRNA transfection, the cells were subjected to real-time PCR.

2.8. Expression of Target Genes in Clinical Specimens and Correlation

In the analysis using surgical specimens (27 paired normal and cancerous tissues), we observed marked suppression of miR-139-3p and marked upregulation of KRT80 in cancer tissues (Figures 1C and S7A). In addition, a negative correlation was observed between the expression of miR-139-3p and KRT80 in CRC specimens (Figure S7B). Contrary to the TCGA data analysis, we did not find any significant upregulation of HK2 in our cancerous samples.

3. Discussion

Because CRC is a heterogeneous disease, as indicated by our genome-wide transcriptome analysis, it is necessary to search for diagnostic markers and therapeutic target molecules in an individualized manner. Recently, we determined the miRNA expression signature of CRC using RNA sequencing [17]. In that study, we found that miR-490-3p acted as a tumor-suppressive miRNA in CRC cells, and expression of its gene targets (IRAK1, FUT1, and GPRIN2) was significantly predictive of 5-year overall survival in CRC.
patients [17]. This new miRNA expression signature of CRC will be a useful tool for elucidating the molecular pathogenesis of this disease.

Aberrant expression of miRNAs is frequently observed in several types of cancers [14–16]. A vast number of studies showed that epigenetic modification (histone modifications and promoter DNA methylation) is closely involved in the silencing of miRNAs expression in cancer cells [23–26]. A recent study showed that miR-139 was epigenetically silenced by histone H3 lysine 27 trimethylation (H3K27me3) in lung cancer cells [25]. Our present data (TSA and 5-aza-dC treatment) showed that both events of histone deacetylation and DNA methylation were closely involved in the silencing of miR-139-3p on CRC cells. It has been shown that miR-139-3p silencing plays a pivotal role in human oncogenesis.

Our recent studies revealed that some passenger strands of miRNAs are closely involved in the molecular pathogenesis of a wide range of human cancers, e.g., miR-30c-2-3p, miR-101-5p, miR-143-5p, and miR-145-3p [19,21,27,28]. Based on our CRC signature, we focused on miR-139-3p (the passenger strand derived from pre-miR-139) in this study. We have analyzed the passenger stand miR-139-3p in several types of cancers and found that it acts as a tumor-suppressive miRNA in bladder cancer, renal cell carcinoma, and head and neck squamous cell carcinoma by targeting several genes closely linked to cancer pathogenesis [29–31]. Here, the function of miR-139-3p in CRC cells was clarified and found to be consistent with previous reports. As we have discussed, our in vitro assays showed that miR-139-3p acted as a tumor suppressive miRNA in CRC cells. However, the endogenous expression levels of passenger strands of miRNAs are little, and the full picture of the functions of passenger strands of miRNAs in vivo remains unknown. In order to investigate the in vivo functions of miRNAs, it is essential to generate and analyze cells that constitutively express miRNAs or cells in which miRNA expression is completely knocked out.

Several oncogenic signaling pathways are activated in CRC cells, of which PI3K/AKT/mTOR signaling is frequently activated [32–35]. Therefore, inhibiting activation of this signaling pathway is an attractive strategy for CRC treatment [32,36–38]. The AKT serine/threonine kinase is activated by phosphatidylinositol-3 kinase (PI3K) or phosphoinositide-dependent kinases via phosphorylation of Thr308 or Ser473 in AKT and activated AKT phosphorylates various downstream protein substrates (e.g., mTOR, glycogen synthase kinase 3 beta, and forkhead box protein O1) [39]. Aberration expression and activation of AKT have been observed in many types of cancers, including CRC [40]. Notably, ectopic expression of miR-139-3p inhibited the phosphorylation of AKT in CRC cells in this study.

Next, we searched for target genes regulated by miR-139-3p in CRC cells, particularly those involved in AKT phosphorylation. A unique feature of miRNAs is that they regulate different sets of genes depending on the cancer cell type. We identified 29 genes as tumor-suppressive targets of miR-139-3p in CRC cells. Of these, we focused on KRT80 because its expression was significantly different between cancer and normal tissues. Ideally, a therapeutic target molecule for cancer is not expressed in normal cells. Expression levels of KRT80 in normal tissues were assessed using previous large-scale transcriptional analysis data [41]. Expression of KRT80 was detected in skin, esophagus, and salivary glands. In contrast, KRT80 was hardly expressed in other tissues (Figure S8).

We showed that aberrant expression of KRT80 enhanced the malignant phenotypes of cancer cells (i.e., proliferation, migration, and invasion). Interestingly, overexpression of KRT80 induced EMT-related genes and activated the AKT signaling through phosphorylation of AKT (Ser 473) [22]. Considering our present data and previous reports, it was strongly suggested that the miR-139-3p/KRT80/p-AKT axis influences the migration and invasive abilities of CRC cells. In ovarian cancer, overexpression of KRT80 induced the expression of genes related to epithelial–mesenchymal transition and activated both MEK and ERK [42]. In gastric cancer, overexpression of the circular RNA CircPIP5K1A induced expression of KRT80 and activated the PI3K/AKT pathway via miR-671-5p adsorption [43]. Moreover, KRT80 expression was significantly correlated with clinical parameters, such as
lymph node metastasis and pathological stage, in CRC and ovarian cancer [22,42]. Together, these data suggest that KRT80 is a potential therapeutic target for CRC.

We also investigated genes affected by KRT80 in CRC cells. In CRC cells, the expression of several genes was suppressed after the knockdown of KRT80 expression. Among these genes, we focused on HK2. The four members of the HK family (HK1-4) in mammals catalyze the conversion of glucose to glucose-6-phosphate, and they are involved in the first and rate-limiting step of glycolysis [44–46]. Previous studies reported that Akt and HK2 are overexpressed in cancer cells and that there is a positive correlation between activation of the PI3K/Akt/mTORC1 pathway and HK2 expression [47–49]. These findings indicate that simultaneous inhibition of glycolysis and the AKT/mTOR signaling pathway is effective in suppressing the growth of cancer cells [50].

4. Materials and Methods

4.1. Clinical Specimens Used to Evaluate miR-139-5p and miR-139-3p Expression

Fifty-four clinical specimens (27 CRC tissues and 27 normal colon tissues) were used to evaluate the expression status of miR-139-5p/3p. All specimens used in this study were obtained by surgical resection at Kagoshima University Hospital between 2014 and 2017. Normal colon tissue was collected from adjacent sites to the specimen from which each CRC tissue sample was taken. All patients provided written informed consent for the use of their specimens. This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Kagoshima University (approval number 160038 (28–65); date of 19 March 2021). The clinical information was described in our previous study [17].

4.2. CRC Cell Lines and Cell Culture

Two CRC cell lines, HCT116 and DLD-1, were used in this study. HCT116 cells were obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), and DLD-1 cells were obtained from the Cell Resource Center for Biomedical Research Bank (Sendai, Miyagi, Japan). HCT116 was cultured in DMEM medium supplemented with 10% concentration of fetal bovine serum (FBS), and DLD-1 was cultured in RPMI-1640 medium, also supplemented with 10% concentration of fetal bovine serum (FBS).

4.3. RNA Extraction and Quantitative Real-Time Reverse-Transcription PCR (qRT-PCR)

The protocols used for RNA extraction and qRT-PCR were described in our previous studies [51,52]. In brief, Total RNA was isolated from cell lines using TRIzol reagent according to the manufacturer’s protocol. RNA samples were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). qPCR was performed using PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and Fast SYBR Grean Master Mix (Applied Biosystems, Waltham, MA, USA), StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). Gene expressions were quantified relatively by the delta-delta Ct method (used GUSB as internal control). TaqMan assays used in this study are summarized in Table S2. The sequences of primers for SYBR green assays are summarized in Table S3.

4.4. Regulation of miR-139-3p Expression by DNA Demethylation

Cells were treated with 5-Aza-2′-deoxycytidine (5-aza-dC; Wako, Osaka, Japan) at concentrations of 0.5, 1.25, and 10 µmol/L for 96 h. Cells were first cultured in growth medium; after 24 h of incubation, the medium was replaced with fresh medium containing 5-aza-dC or Dimethyl sulfoxide (DMSO, negative control), and cells were incubated for another 48 h; after 48 h of treatment, the medium was again replaced with fresh medium containing 5-aza-dC or DMSO and cells were cultured for additional 48 h. After 120 h treatment, total RNA was isolated. The expression levels of miR-139-3p were measured by qRT-PCR.
4.5. Regulation of miR-139-3p Expression by Histone Deacetylation

Cells were treated with Tricostatin A (TSA; Wako, Osaka, Japan) at 0.1 or 0.5 µmol/L concentration for 24 h. Cells were first grown in growth medium. After 24 h of incubation, the medium was replaced with fresh medium containing TSA or DMSO and the cells were incubated for an additional 24 h. After 48 h treatment, total RNA was isolated. Expression levels of miR-139-3p were measured by qRT-PCR.

4.6. Transfection of miRNAs and siRNAs into CRC Cells

The protocols used for transient transfection of miRNAs and siRNAs were described in our previous studies [51,52]. The miRNA precursors and siRNAs used in this report were detailed in Table S2. Opti-MEM (Gibco, Carlsbad, CA, USA) and Lipofectamine™ RNAiMax Transfection Reagent (Invitrogen, Waltham, MA, USA) were used for miRNA and siRNA transfection of miRNAs and siRNAs into CRC cell lines. All miRNA precursors and siRNAs were transfected into the CRC cell line at 10 nM. Mock transfection consisted of cells without precursors or siRNAs. Control groups were transfected with the negative control precursor.

4.7. Functional Analyses (Tumor Suppression and Promotion Assays) in CRC Cell Lines

The tumor-suppressive functions of miRNAs were evaluated by transient transfection assays using mature miR-139-5p and miR-139-3p. The tumor-promoting functions of KRT80 (loss-of-function assays) were assessed by siRNA transfection assays using siRNAs targeting KRT80. Functional assays (proliferation, migration, and invasion assays) were performed according to procedures of previous studies [51,52]. Briefly, for proliferation assays, HCT116 or DLD-1 cells were transferred into 96-well plates at 3.0 × 10^5 cells/well. Cell proliferation was assessed using XTT assay kit II (Sigma-Aldrich, St. Louis, MO, USA) 72 h after the transfection procedure. For the migration and invasion assay, HCT116 and DLD-1 cells were transfected in 6-well plates at 3.0 × 10^5 cells/well; 48 h later, transfected HCT116 and DLD-1 cells were added to each chamber at 1.0 × 10^5 cells/well. Corning BioCoat™ cell culture chambers (Corning, Corning, NY, USA) were used for the migration assay and Corning BioCoat Matrigel Invasion Chambers were used for the invasion assay. Cells on the underside of the chamber membrane were stained and counted for analysis. All experiments were performed in triplicate. The details of the reagents used in these analyses are listed in Table S2.

4.8. Identification of Putative Targets Regulated by miR-139-3p in CRC Cells

To identify oncogenic targets controlled by miR-139-3p in CRC cells, data were merged from the following sources to narrow down the targets: (1) Target Scan Human 8.0 database (http://www.targetscan.org/vert_80, accessed on 6 August 2021) [53], (2) gene expression data from miR-139-3p transfected CRC cells (GEO accession number, GSE155659), and (3) gene expression database from CRC clinical tissues using the GEPIA2 platform (http://gepia2.cancer-pku.cn/#index; accessed on 10 April 2022) [54].

4.9. RIP Assay

The assay for RIP was performed according to previous studies [55]. Briefly, CRC cells were cultured in 6-well dish at 3.0 × 10^5/well concentration. Negative control miRNA precursors and miR-139-3p precursors were transfected. After 12 h, immunoprecipitation was performed using the MagCapture™ microRNA Isolation Kit, Human Ago2, obtained from FUJIFILM Wako Pure Chemical Corporation (Wako, Osaka, Japan) according to the manufacturer’s protocol. Expression levels of KRT80 and HK2 bound to Ago2 were measured by qRT-PCR. TaqMan assays used in this study are summarized in Table S2. The sequences of primers for SYBR green assays are summarized in Table S3.
4.10. Dual-Luciferase Reporter Assay

The dual-luciferase reporter assay was performed to determine whether miR-139-3p binds directly to the 3′-UTR of KRT80. A partial wild-type sequence, including the seed sequence, of the KRT80 3′-UTR, was inserted into the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Alternatively, the same KRT80 3′-UTR sequence but with the miR-139-3p binding site deleted was also inserted into the same vector to create the deletion-type construct. The design of each vector cloning sequence into wild-type and deletion-type were shown in Figures S9 and S10. mRNA sequences of KRT80 and HK2 were cited from National Center for Biotechnology Information database [56]. The dual-luciferase reporter assay was performed according to previous studies [17,52]. The reagents used in the assay are listed in Table S2.

4.11. Western Blot and Immunohistochemical Analyses

The procedures for Western blot and immunohistochemical analyses were performed according to our previous studies [51,52]. In brief, 72 h after transfection, cells were collected, and lysates were prepared. Next, 18 µg/lane of protein lysate was separated on e-PAGE (ATTO, Tokyo, Japan), transferred to PVDF membranes, and incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. GAPDH was used as an internal control. The antibodies used are listed in Table S2, and the clinical specimens evaluated by immunohistochemistry are shown in Table S4.

4.12. Statistical Analyses

JMP Pro 15 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analyses. Differences between two groups were assessed using Welch’s t-test and those among multiple groups using Dunnett’s test. Spearman’s test was used for the correlation analyses. A p-value less than 0.05 was considered statistically significant.

5. Conclusions

Based on the miRNA expression signature of CRC obtained by RNA sequencing, the expression of miR-139-3p (the passenger strand) was significantly reduced in CRC tissues. Functional assays revealed that expression of miR-139-3p attenuated cancer cell malignant phenotypes, indicating that miR-139-3p acts as a tumor suppressor in CRC cells. KRT80 was identified as a direct target of miR-139-3p, and aberrant expression of KRT80 was confirmed in CRC clinical specimens. Moreover, HK2 expression was regulated by both miR-139-3p and KRT80 in CRC cells. Exploration of miRNA-regulated molecular networks provides important information for identifying therapeutic targets for CRC.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Kagoshima University (approval no. 160038 28-65, date of approval: 19 March 2021).

Informed Consent Statement: Written prior informed consent and approval were obtained from all patients who were diagnosed with CRC and resected at Kagoshima University Hospital.
**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

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