MicroRNA-17-5p regulates EMT by targeting vimentin in colorectal cancer

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BACKGROUND: Epithelial–mesenchymal transition (EMT) is the most common cause of death in colorectal cancer (CRC). In this study, we investigated the functional roles of miRNA-17-5p in EMT of CRC cells.

METHODS: In order to determine if miRNA-17-5p regulated EMT, the precursors and inhibitors of miR-17-5p were transduced into four CRC cells. To evaluate the regulatory mechanism, we performed argonaute 2 (Ago2) immunoprecipitation (IP) and luciferase assay. In addition, we used an intra-splenic injection mouse model of BALB/c nude mice to investigate the metastastic potential of miRNA-17-5p in vivo.

RESULTS: The miRNA-17-5p expression was lower in primary CRC tissues with metastasis than in primary CRC tissues without metastasis in our RNA sequencing data of patient tissue. Real-time quantitative PCR revealed that miRNA-17-5p was inversely correlated with that of vimentin in five CRC cell lines. Over-expression of miRNA-17-5p decreased vimentin expression and inhibited cell migration and invasion in both LoVo and HT29 cells. However, inhibition of miRNA-17-5p showed the opposite effect. Ago2 IP and luciferase assay revealed that miRNA-17-5p directly bound to the 3′UTR of VIM mRNA. Furthermore, miRNA-17-5p inhibited the metastasis of CRC into liver in vivo.

CONCLUSIONS: Our results demonstrated that miRNA-17-5p regulates vimentin expression, thereby regulating metastasis of CRC.

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**METHODS**

Cell culture and transfection

Colorectal cancer cell lines (HCT15, Colo205, LoVo, HT29, SW480, HCT116, DLD-1, SW620, SW48, LS113, and RKO) were purchased from American Type Culture Collection (ATCC) and cultured with RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) in a 37 °C incubator with 5% CO₂. For transient transfection, cells were transfected with indicated siRNA or miRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. siRNAs and miRNAs used in this study were as follows: control miRNA (4464058, Applied Biosystems), and vimentin siRNA (sc-29522, Santa Cruz Biotechnology, CA, USA), miR-17-5p mimic (4464066, Applied Biosystems), and miR-17-5p inhibitor (4464084, Applied Biosystems). All experiments were performed with mycoplasma-free cells.

Cell lysis and western blot analysis

To prepare whole-cell extract, cells were lysed using Pro-prep buffer (Intron Biotechnology, Seoul, Korea) including protease inhibitors. Similar amounts of protein extracts were resolved via SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were probed with primary antibodies followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, CA, USA). β-actin was used as a loading control in western blot analysis. Antibodies used for this study were as follows: E-cadherin (24E10) (#3195, Cell Signaling Technology, Danvers, USA), vimentin (#MA5-11883, Thermo Fisher Scientific, MA, USA), and β-actin (#3700, Cell Signaling Technology).

Cell migration and invasion assays

Cell lines were seeded onto six-well plates. When the cell confluence reached about 80% and above at around 48 h post transfection, scratch wounds were made by scraping the cell layer across each culture plate using the tip of a 10 μl pipette. After wounding, the debris was removed by washing the cells with phosphate buffered saline (PBS). Wounded cultures were incubated in serum-free medium for 30 h, and then three fields (10×) were randomly picked from each scratch wound and visualised by microscopy to assess cell migration ability. The experiments were performed in triplicate.

Cell invasion assays were carried out using a Matrigel invasion chamber (Corning, USA) hydrated for at least 2 h with 500 μl Hanks balanced salt solution (HBSS) (Gibco) and injected into a mouse spleen with a 30-gauge needle. To prevent tumour cell leakage and bleeding, the incision was made, and the spleen was exteriorised for intra-splenic injection. LoVo cells with NC-mimic (Control group, 1.2 × 10⁶ cells) were transfected with indicated siRNA or miRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and protease K (Bioneer, Daejeon, South Korea). Proteins were denatured with acid phenol (Ambion) and RNAs were precipitated with absolute ethanol overnight at –20 °C. The level of mRNA in AgO2-mediated RNA-induced silencing complex (RISC) was determined by RT-qPCR as described above.

Luciferase assay

To determine the miRNA recognition elements in the 3’UTR of VIM mRNA, we prepared four luciferase reporter constructs as follows: (A) blank pmirGLO vector, (B) wild-type 3’UTR of vimentin mRNA (GTTCCCTCTAAACCCTAGAGGAGC), (C) mutant 3’UTR of vimentin mRNA in the miR-17-5p seed region (CCTTTGTCTAAACCCTAGAGG), and (D) perfect match in the miR-17-5p seed region (GTTTACCTTAAACCCTAGAGG). Luciferase activities were measured using a luminometer according to the manufacturer’s instructions (GloMax20/20 luminometer, Promega) 24 h post transfection using the Dual-Glo luciferase activity assay system (Promega). Renilla luciferase activity was normalised using firefly luciferase activity for each sample.

Intra-splenic injections

Six- to seven-week-old female BALB/c nude mice (Provided Orient- Bio in Korea) were anesthetised with a mixture of Catamin (#7001, Yuhan) (30 mg/kg) and Rompun (Bayer) (10 mg/kg) via intra-peritoneal injection (0.01 ml/kg). A small left abdominal flank incision was made, and the spleen was exteriorised for intra-splenic injection. LoVo cells with NC-mimic (Control group, 1.2 × 10⁶ cells) and LoVo cells with miR-17-5p mimic (Experimental group, 1.2 × 10⁶ cells) were suspended in 50 μl Hanks’ balanced salt solution (HBSS) (Gibco) and injected into a mouse spleen with a 30-gauge needle. To prevent tumour cell leakage and bleeding, a cotton swab was held over the site of injection for 1 min. The injected spleen was returned to the abdomen and the wound was sutured with 6-0 black silk.

When we conducted the sacrifice, we placed the mice in the chamber and introduced 100% carbon dioxide. After we observed each mouse for lack of respiration and faded eye colour, we maintained the carbon dioxide flow for a minimum of 1 min after respiration ceases.

When female BALB/c nude mice were 6- to 7-week-old, we carried out intra-splenic injection. After 6 weeks, we checked the MRI image and sacrificed the mice for obtaining tissue.

The animal experiments were tested in specific pathogen-free animal experiments centre at the Samsung Medical Center.

To establish whether miR-17-5p activity was related to metastasis in vivo, we developed a metastatic mouse model. The intra-splenic injection model displayed the fastest approach to develop a liver metastasis mouse model than caecal-injection mouse model. According to our previous research, these anaesthesia methods can minimise the side effects and death, and we also used local anaesthetics after surgery to further reduce the pain.

Ethics approval for animal use was obtained from the Samsung Medical Center on Laboratory Animals Committee (approval number: 20180129002). We carried out animal experiments in accordance with the ARRIVE reporting guidelines (attached supplementary file).
miR-17-5p expression is inversely correlated with the expression of vimentin in colon cancer cells. In order to investigate the expression of specific miRNAs of CRC, we obtained RNA and miRNA sequencing data from 43 CRC patients. We found that 29 CRC patients showed liver metastasis and 14 patients did not. Analysis of RNA sequencing data of patient tissue indicated that the miR-17-5p expression was lower in primary CRC tissues with metastasis than in primary CRC tissues without metastasis (Fig. 1a). To determine if miR-17-5p was involved in the EMT process, the levels of E-cadherin and vimentin were analysed by western blot in 11 colon cancer cell lines (Fig. 1b). Furthermore, disease-free survival analysis of TCGA data indicated that the low-level group of miR-17-5p showed poor prognosis compared to the high-level group (Supplementary Fig. S1B, $P = 0.0189$) and the high-level group of VIM expression showed poor prognosis than the low-level group (Supplementary Fig. S1C, $P = 0.0386$). The high group of each gene is the top 30% of all patients in gene expression, and the low group is the bottom 30% of all patients. Based on the expression of vimentin, five colon cancer cells were selected for further studies: (1) low-expressing group: HCT-15 and Colo205; (2) medium-expressing group: HT29 and LoVo; and (3) high-expressing group: SW480. RT-qPCR analysis showed that the expression of vimentin in LoVo, HT29, and Colo205 cells were selected for further studies: (1) low-expressing group: HCT-15 and Colo205; (2) medium-expressing group: HT29 and LoVo; and (3) high-expressing group: SW480. RT-qPCR analysis showed that the expression of vimentin in LoVo, HT29, and Colo205 cells was increased in the following order: HCT-15 < Colo205 < HT29 < LoVo < SW480 (Fig. 2a). Furthermore, disease-free survival analysis of TCGA data indicated that the low-level group of miR-17-5p showed poor prognosis compared to the high-level group (Supplementary Fig. S1B, $P = 0.0189$) and the high-level group of VIM expression showed poor prognosis than the low-level group (Supplementary Fig. S1C, $P = 0.0386$). The high group of each gene is the top 30% of all patients in gene expression, and the low group is the bottom 30% of all patients. Based on the expression of vimentin, five colon cancer cells were selected for further studies: (1) low-expressing group: HCT-15 and Colo205; (2) medium-expressing group: HT29 and LoVo; and (3) high-expressing group: SW480. RT-qPCR analysis showed that the expression of vimentin in LoVo, HT29, and Colo205 cells was increased in the following order: HCT-15 < Colo205 < HT29 < LoVo < SW480 (Fig. 2a). In contrast, down-regulation of miR-17-5p by transducing miRNA inhibitor increased the expression of vimentin in LoVo, HT29, and Colo205 cells (Fig. 2c). The level of VIM mRNA was slightly increased via down-regulation of miR-17-5p (Fig. 2d). Based on these results, we speculated that miR-17-5p regulated the vimentin expression.
miR-17-5p directly interacts with the 3′ UTR of VIM mRNA. Based on the above results, we found that miR-17-5p suppresses vimentin expression. Therefore, we tested if miR-17-5p directly binds to the 3′ UTR of VIM mRNA via Argonaute 2 (Ago2) ribonucleoprotein immunoprecipitation (RNP-IP). Ago2 RNP-IP revealed that VIM mRNA was more enriched in miR-17-5p-overexpressed LoVo and HT29 cells when compared with control miRNA, indicating that miR-17-5p directly binds to the 3′ UTR of VIM mRNA. Representative results are shown in Fig. 3a and the results obtained from three independent experiments are presented in Supplementary Fig. S3. Direct interaction between miR-17-5p and the 3′ UTR of VIM mRNA was subsequently assessed via luciferase-based assay. Interestingly, VIM mRNA is not predicted as a target mRNA of miR-17-5p in any prediction programs. However, we realised that only a single nucleotide of the 3′ UTR of VIM mRNA was mismatched to the seed sequence of miR-17-5p (Fig. 3b). We constructed three luciferase reporter plasmids containing (1) wild-type 3′ UTR of VIM mRNA harbouring a single nucleotide mismatch to the seed sequence of miR-17-5p (Fig. 3b, upper), (2) mutant type 3′ UTR of VIM mRNA to block the binding of miR-17-5p (Fig. 3b, middle), and (3) mutant type 3′ UTR of VIM mRNA which is perfectly matched to the seed sequence of miR-17-5p mimic, and the luciferase activity was determined. The luciferase reporter assay showed that overexpression of miR-17-5p suppressed luciferase expression in the wild-type and perfectly matched reporters. However, the luciferase expression was not influenced by mutant reporters (Fig. 3d). These results indicate that even a single nucleotide difference in the miRNA recognition element (MRE) led to target gene suppression by miR-17-5p. Furthermore, our findings suggest that target genes of miRNA are much more extensive than those predicted.
miR-17-5p induces inhibition of cell migration and invasion

To evaluate the role of miR-17-5p in controlling the metastatic potential of colon cancer, migratory and invasive activities were assessed by wound closure assay and Transwell invasion assay, respectively. In the wound closure assay, we found that miR-17-5p inhibited the migratory ability of LoVo and HT29 cells that was the same effect in VIM-silenced cells (Fig. 4a, b, respectively). Conversely, the decrease of miR-17-5p by transducing miRNA inhibitor enhanced the migratory ability of LoVo and HT29 cells (Fig.4c, d, respectively). Invasiveness of colon cancer cells was also influenced by miR-17-5p. In LoVo cells, we observed that miR-17-5p inhibited invasive activity (Fig. 4e). Similarly, the number of invaded cells was decreased in VIM-silenced cells. However, the decreased expression of miR-17-5p showed higher invasiveness compared with control (Fig. 4f). These results demonstrate that miR-17-5p controlled the metastatic potential including migration and invasion of colon cancer cells by suppressing vimentin expression.

miR-17-5p inhibits metastasis in mouse model

Based on the foregoing results, it is clear that miR-17-5p inhibits CRC metastasis in vitro. Additionally, to establish whether miR-17-5p activity was related to metastasis in vivo, we developed a metastatic mouse model by injecting miR-17-5p overexpressing LoVo cells. Seven BALB/c-nude mice were used in each group. Fewer metastases were found in the miR-17-5p overexpressing group injected with LoVo cells (14%, 1 out of 7 mice) than in the control group (71%, 5 mice out of 7). We collected metastatic tumour tissues and analysed the tissue histology to confirm tumour metastasis and detected the expression of miR-17-5p and Vimentin as a mesenchymal marker. Examination of metastatic tumours by H&E staining revealed liver metastatic nodules in the control group injected with LoVo cells (Fig.5a, left panel), which were the same mice in Fig.5b (left panel) but not in mice injected with miR-17-5p overexpressing LoVo cells (Fig. 5a, right panel). MRI image revealed liver metastasis in control LoVo-injected mice during the monitoring period (Fig.5b, left panel). However, no liver metastasis was detected on MRI in overexpressing miR-17-5p-injected LoVo cells (Fig.5b, right panel). Increased expression of miR-17-5p compared with the control group was observed in the miR-17-5p overexpressing group (Fig. 5c, left panel). In addition, Vimentin expression was reduced in the miR-17-5p overexpressing group (Fig. 5c, right panel). Furthermore, to establish whether vimentin expression was related to metastasis in vivo, we developed a metastatic mouse model by injecting control LoVo cells and Vimentin-down-regulated LoVo cells (Supplementary Fig. S4A). Five BALB/c-nude mice were used in each group. Fewer metastases were found in
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Additionally, our data indicated that miR-17-5p bound vimentin expression, cell migration and invasion in vitro. Moreover, our findings suggested that overexpression of miR-17-5p inhibited liver metastasis in an intra-splenic injected mouse model. We also found that miR-17-5p overexpression in CRC cell lines increased vimentin protein expression, cell migration, and invasion, whereas down-regulation significantly decreased VIM mRNA expression, vimentin protein expression, cell migration, and invasion, whereas down-regulation of miR-17-5p in CRC cell lines increased vimentin protein expression, cell migration and invasion in vitro. Moreover, our findings suggested that overexpression of miR-17-5p inhibited liver metastasis in an intra-splenic injected mouse model. Additionally, our data indicated that miR-17-5p bound vimentin 3′UTR and regulated its expression. Therefore, our study indicated a new molecular biomarker for CRC prognosis.

**DISCUSSION**

MiRNAs have been implicated in CRC tumorigenesis and metastasis, and represent effective molecular biomarkers for CRC diagnosis, prognosis, and therapy. In this study, we found that miR-17-5p expression was significantly down-regulated in primary CRC tissues with metastasis than in those without metastasis in our sequencing data, and its expression was inversely correlated with vimentin expression in CRC tissues and cell lines. In addition, we also found that miR-17-5p overexpression in CRC cell lines significantly decreased VIM mRNA expression, vimentin protein expression, cell migration, and invasion, whereas down-regulation of miR-17-5p in CRC cell lines increased vimentin protein expression, cell migration and invasion in vitro. Moreover, our findings suggested that overexpression of miR-17-5p inhibited liver metastasis in an intra-splenic injected mouse model. Additionally, our data indicated that miR-17-5p bound vimentin 3′UTR and regulated its expression. Therefore, our study indicated a new molecular biomarker for CRC prognosis.

Using RNP Ago2 IP and luciferase reporter assays, we identified vimentin 3′UTR as the new direct miR-17-5p downstream target. Although we established that miR-17-5p regulated vimentin expression, we found no specific target sequences in the prediction program, which only showed a perfect target match with miRNA seed. No perfect match was found between vimentin 3′UTR and miR-17-5p seed. We assumed a mismatch between miR-17-5p and vimentin based on Ago2 IP. The miRNAs exhibit six primary binding patterns defined according to the position of nucleotide binding between the miRNA and target mRNA. The seed mismatch occurs without adenosine in the binding position, and the imperfect centred match refers to the evolutionary conservation of the central region of miRNAs. Imperfect binding within the seed region can be resolved by a high degree of complementary binding at the 3′ end of the miRNA, facilitating the overall binding energetics of the miRNA-target. However, the seed matches are not currently shown by miRNA, facilitating the overall binding energetics of the miRNA-target.

**Fig. 4** Control of cell migration and invasion by miR-17-5p. a, b Over-expression of miR-17-5p and silencing of vimentin decreased LoVo and HT29 cell mobility. Left: The width of the scratch-wounded cell monolayer was recorded at 0 and 24 h, or 0 and 30 h after wounding via photography. Right: The relative migration distance presented in the column chart. c, d Down-regulation of miR-17-5p increased cell migration in LoVo and HT29. e Over-expression of miR-17-5p and silencing of vimentin inhibited LoVo cell invasion based on Transwell assays. f Down-regulation of miR-17-5p increased cell invasion in LoVo. The bars represent mean ± SD. *P < 0.05. All the experiments were repeated three times.

The vimentin down-regulated group (0%, 0 mice out of 5) than the control group (80%, 4 mice out of 5) (Supplementary Fig. S4B). These data suggested that miR-17-5p reduced liver metastasis by targeting vimentin in vivo.
8-mers (bases 1–8). However, the prediction program did not show 7-mer-A1 and 8-mer. Therefore, we found the 6–8 mer with and without mismatch and evaluated the three binding sites via a luciferase assay. The first binding site, which was a 7-mer-A1 with 1 mismatch, reduced luciferase activity compared with the control. Although wild-type binding site was associated with partially reduced luciferase activity, the mutant site was restored. Additionally, a perfect match reduced the binding by a similar percentage compared with the wild type. Therefore, miR-17-5p bound to vimentin 3′UTR in spite of a binding mismatch.

In this study, we confirmed that miR-17-5p regulated EMT by binding vimentin 3′UTR in CRC. It has been reported that miR-17-5p is up-regulated in osteosarcoma and retinoblastoma, but acts as a metastatic inhibitor in non-small-cell lung cancer and breast cancer. Although miR-17-5p has varying function in each cancer type, it might be a metastatic suppressor or oncogene in an organ-dependent manner. Additionally, miR-17-5p has yielded controversial results in CRC. However, the reported studies and our results used different CRC cell lines and target genes. The binding site sequence of the target genes may differ due to point mutations in each cell line, and the binding force of miRNA may also vary. Because miRNAs exhibit different seed types and binding mismatch, they bind to different target genes in different cell lines and organs. MiR-17-5p is a representative miRNA with several binding types. Therefore, the differences in miR-17-5p expression and their contrasting effects in each cancer and CRC deserve further investigation.

Jiang et al. reported that up-regulated miR-17–92 acted as a negative regulator of activation in the Wnt/β-catenin pathway and inhibited CRC metastasis. The study showed that the overexpression of miR-17–92 reduced vimentin expression; however, the reduction of vimentin was regulated via inhibition of Wnt/β-catenin pathway by miR-17–92. In our data, miR-17-5p directly targeted vimentin 3′UTR and reduced vimentin expression. Therefore, miR-17-5p regulated EMT via inhibition of Wnt/β-catenin pathway as well as by directly targeting vimentin.

In conclusion, this study demonstrates that miR-17-5p regulates EMT by targeting vimentin in CRC. However, there are many targets of miR-17-5p other than vimentin. MiR-17-5p might regulate EMT by targeting other genes as well as vimentin. Six EMT-related genes, which are targeted by miR-17-5p, were found from Targetscan. The six genes are STAT3, E2F1, HMGA2, SOX4, TWIST1, and EGFR that have been studied to be associated with EMT of CRC. Although it has not been studied that the genes are regulated by miR-17-5p in CRC, miR-17-5p might regulate EMT by targeting these EMT-related genes. These findings provide novel insights into the molecular mechanisms of CRC metastasis. Therefore, this study might facilitate the development of new therapeutic strategies and novel biomarkers.

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AUTHOR CONTRIBUTIONS

T.W.K., Y.S.L., and Y.B.C. conceived the study design. T.W.K., Y.S.L., N.H.Y., C.H.S., and H.K.H. conducted the experiments. T.W.K. wrote the initial manuscript drafts. Y.S.L., H.H.K., and Y.B.C. performed critical editing. All authors approved the final version of the manuscript.
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ADDITIONAL INFORMATION

Ethics approval and consent to participate
Ethics approval for animal use was obtained from the Sungkyunkwan University Animal Ethical Committee (approval number: 20180124006). We carried out animal experiments in accordance with the ARRIVE reporting guideline and Sungkyunkwan University’s institutional guidelines.

Data availability
The data supporting the findings of this study are available within the article and are available from the corresponding authors upon request.

Competing interests
The authors declare no competing interests.

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Supplementary information
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